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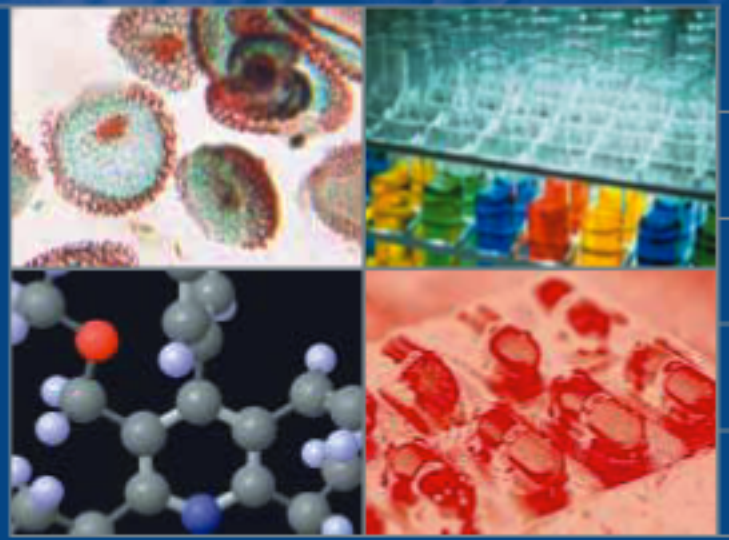
Drug Discovery and Evaluation

Pharmacological Assays

Second Edition



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H. Gerhard Vogel (Ed.)

Drug Discovery and Evaluation

Pharmacological Assays

Second Completely Revised, Updated, and Enlarged Edition

Including a CD-ROM

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Preface to the second edition

The first edition of "Drug Discovery and Evaluation – Pharmacological Assays" has been well accepted by a broad readership ranging from experienced pharmacologists to students of pharmacology. Therefore, already after a short period of time the question for a second edition arose.

The first edition was mainly centered around the personal laboratory experience of the editors and the contributors. The second edition tries to close evident gaps.

The input of biochemistry to pharmacology has grown. Molecular pharmacology puts more emphasis on the mode of action of drugs, albeit it becomes clear that the activities of most drugs are not confined to one single mode of action. Studies in single cells become more and more popular, however, they do not cover the complexity of a whole organism. Possible side effects of drugs can be better detected in whole animals than in single cells. Therefore, the new requirements of the health authorities on safety pharmacology put emphasis on experiments not only in whole anesthetized animals but in conscious ones. The second edition of this book takes note of these requirements and devotes special chapters for each indication to safety pharmacology.

Molecular biology also introduced new methods to pharmacology, such as the polymerase chain reaction (PCR), reverse PCR, Northern, Western and Southern blotting. Very recently, microarray technology, proteomics, and mass spectroscopy were added as novel *in vitro* methods. Furthermore, genetically modified animals have been created which resemble human diseases. Pharmacogenomics has already begun to influence pharmacology and even will have greater input in the future. Special attention is given to these new achievements in various chapters of the book.

The editor and the co-editors are well aware that the rapid progress in biology during the next decades will change the methodological approach. Electronic media will help the researcher for continuous information. However, it becomes more and more evident, that young pharmacologists have only insufficient training in the classical pharmacological methodology. Searching for these methods, e.g., for safety pharmacology, the researchers cannot find sufficient information on the methodological details in the electronic data bases currently available. This book covering the pharmacological methods of more than hundred years may be of help.

The guidelines concerning the care and use of laboratory animals have been updated.

A change in paradigm of pharmacological research has been claimed but superiorities of these new approaches compared to the old ones have still to be proven. To address this, an introductory chapter on new strategies in drug discovery and evaluation has been added, including combinatorial chemistry; high throughput screening, ultra high throughput screening and high content screening; pharmacogenomics, proteomics and array technology. Some critical thoughts on errors in screening procedures have been added.

At this place, we would like to express our sincere thanks to all colleagues who contributed to the new and to the first edition of this book. Their names and their positions are given alphabetically.

March 2002

H. Gerhard Vogel
Also in the name of the co-editors

Preface to the first edition

This book is intended to be an aid for experienced pharmacologists as well as for newcomers in the field of experimental pharmacology. The student in pharmacology, the pharmacist and the medicinal chemist will find a survey of pharmacological assays that can be used for a given indication and for which methods have demonstrated their relevance. The researchers working in special fields of pharmacology will find assays in other, unfamiliar areas which might help to expand their own research.

Certain therapeutic domains, such as cardiovascular, respiratory and renal disorders, psychiatry and neurology, peripheral nerve function, pain and rheumatic diseases, metabolic and endocrine diseases including diseases of the gastrointestinal tract, are discussed in this book.

Each chapter is divided into pharmacological classes, e.g., anxiolytics, anti-epileptics, neuroleptics, antidepressants, or anti-Parkinson drugs. For each class, in vitro methods, tests on isolated organs and in vivo methods are described.

For each method the purpose and rationale are given first, followed by a description of the procedure, evaluation of the data, modifications of the method described in the literature, and the relevant references. If possible, a critical assessment of the method based on personal experience is added. The hints for modifications of the method and the extended reference list will be of value for the experienced pharmacologist.

A few words for the justification for a book of this kind:

In 1959, A. J. Lehman, Director of the Division of Pharmacology at the Food and Drug Administration, USA, wrote:

> … Pharmacologists are individualists. Like most scientists they are seldom willing to copy each other's techniques in detail, and so their methods vary from one to the other. Nevertheless, there are basic principles and techniques which must be applied to establish the safety of a new drug.

Visitors could also read a sticker in his office:

> You too can learn pharmacology, in only three lessons: each of them lasting ten years.

Pharmacologists have always used methods from neighboring disciplines; in the past, e.g. from anatomy, pathology, surgery, zoology and predominantly physiology. Useful methods also came from electrophysiology and the behavioral sciences. Earlier drug discovery was almost exclusively based on animal experiments, clinical observations and serendipity.

In recent years, a major input has come from biochemistry. The effect of many drugs in human therapy could be explained biochemicaly as effects on specific enzymes or receptors. With the detection of more and more receptor subtypes, the activity spectrum of a single compound became more and more complicated. At present, molecular biology provides pharmacologists with human receptors and ion channels expressed in mammalian cells in culture. This avoids the apparently existing species differences, but the multitude of natural and perhaps artificial subtypes raises the question of physiological and pathological relevance.

The challenge for the pharmacologist always will be to correlate in vitro data with in vivo findings, bearing in mind the old saying: "*In vitro simplicitas, in vivo veritas*". The effects found in tissue cultures are quite often not typical for an intact organism.

Pharmacologists, especially in industry, have the task to find new drugs for human therapy by using appropriate models. Pharmacological models have to be relevant, that means they should predict the intended therapeutic indications. A pharmacological model can be considered relevant or correlational, if the effects obtained correlate with results observed in human therapy.

To be relevant or "correlational", a model has to fulfill some basic criteria:

- First, the model must be sensitive in a dose-dependent fashion to standard compounds that are known to possess the desired therapeutic property.
- Second, the relative potency of known active agents in the model should be comparable to their relative potency in clinical use.
- Third, the model should be selective, i.e. the effects of known agents in this therapeutic indication should be distinguishable from effects of drugs for other indications. Positive data with a new compound allow the prediction of a therapeutic effect in patients.

If new assays are applied to indications for which no effective drug is known, there must be sufficient evidence that this model is relevant for the pathological status in this indication.

The methods presented in this book have been selected according to these criteria.

Considerable discussion is going on about the necessity of animal experiments. One has to accept that only the whole animal can reflect the complexity of a human being. Even an experiment with human volunteers is only a model, albeit a highly relevant one, to investigate therapeutic effects in patients. The degree of relevance increases from isolated molecules (e.g. receptors or enzymes) to organelles, to organs up to conscious animals and human volunteers.

Without any doubt, animal experiments are necessary for the discovery and evaluation of drugs. However, they should be performed only if they are necessary and well conceived.

In Chapter N, regulations existing in various countries concerning the care and use of laboratory animals are listed. Furthermore, guidelines for anesthesia, blood collection and euthanasia in laboratory animals are given. In carrying out animal experiments, one must adhere strictly to these guidelines. Following these rules and planing the experiments well, will eliminate or minimize pain and discomfort to the animal. The methods described in this book had the welfare of the animals as well as the benefit of the procedure for the well-being of mankind in mind.

Here, we would like to express our sincere thanks to all colleagues who contributed to this book. Their names and positions are given alphabetically below.

Autumn 1996

H. Gerhard Vogel
Wolfgang H. Vogel

Editor and co-editors

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Contents

Introduction
Strategies in drug discovery and evaluation[1]

0.1 Historical approaches in drug discovery

Today's medicine is based on traditional medicine. Traditional medicines exist in every continent of the globe and in every cultural area of the world. The most famous ones are traditional Chinese medicine in East Asia, Ayurvedic medicine in India, and formerly Galenic medicine in Europe, having same resemblance to each other (Vogel 1991).

Each of these traditional medicines has its own origin and an individual basic philosophy. The art of practicing **Chinese medicine** stretches back over several thousand years. The legendary culture hero, Shen-nong, is said to have tested many herbs for their medical properties. *Pen-ts'ao*, the first compilation of herbal medicines, is connected with his name (Unschuld 1973, 1986). Since ancient times, the Chinese have divided the world into five symbolic elements: Wood, Fire, Earth, Metal and Water. Everything in the world is dominated by one of these elements, and their constant interplay, combined with those of yin and yang, explain all change and activity in nature. The positive, generative cycle proceeds as follows: Wood burns to generate Fire. Fire produces ashes which generates Earth. Earth generates Metal which can be mined from the ground. When heated Metal becomes molten like Water, Water promotes growth of plants, thereby generating Wood. The negative subjugative cycle is complementary to the positive, generative one. Chinese medical views regarding the vital internal organs are based on the theories of *yin* and *yang*, the five elements which are each related to body organs and colors, and the meridians. With medicines consisting mainly of herbal drugs and minerals, Chinese doctors manipulate these natural relationships to adjust energy imbalances caused by the excess or deficiency of these forces in the body. Chinese physicians and philosophers developed a special system of physiology describing vital organs as storage houses and vital connections as meridians that became the basis of acupuncture (Porkert 1973).

In India *Ayurveda*, *Siddha* and *Unani* systems of medicine provide health care for a large part of the population. The word **Ayurveda** is composed of two parts: Ayu (= life) and Veda (= knowledge). The origins of this science of life had been placed by scholars of Ayurveda at somewhere around 6000 BC. They were orally transmitted by successive generations. The principles were recorded in great detail in compendia which are called *Samhitas* (Dash and Junius 1987; Dahanukar and Thatte 1989; Mazars 1990). Compared with modern anatomy and physiology Ayurveda is based on certain fundamental doctrines, known as the *Darshanas*, such as the seven *Dhatus*. They can be described not exactly as organs but as body constituents. The three *Doshas: vata*, *pitta* and *kapha* are regulators of cell function in various ways. A balance of the three doshas is essential for maintaining health. Imbalance of the doshas creates disease. Drug therapy in Ayurveda is highly individualized. The choice of drugs as well as the dose is not only influenced by the disease process, but also by the constitution of individuals and environmental conditions which affect the balance of the doshas and therefore the response to drugs.

Both traditional Chinese and Ayurvedic medicine developed further in terms of formulations. There is

[1] With contributions by G. Rieß and W. F. Vogel.

also the tendency to adopt the modern forms of clinical trials. But there has never been a change in paradigm as far as the basic philosophy is concerned.

Traditional European medicine goes back to the time of Egyptian and Babylonian-Assyrian culture. About 3 000 years BC the Sumerians developed a system of cuneiform writing that enabled them to write on clay tablets. At the time of King Assurbanipal 2 000 years BC a first comprehensive Materia Medica was written containing approximately 250 vegetable drugs and 120 mineral drugs (Koecher 1963). Around 1500 BC, the famous Egyptian papyrus *Ebers* was written, describing more than 700 drugs drawn from plants, animals and minerals. Some are still used in our time, such as garlic and poppy seeds.

Greek philosophers such as Empedocles, Aristoteles and Pythagoras, all from around 500 BC, influenced European medicine to a great deal. They created the theory of the Four Elements which were proposed to be the components of all matter, including animal and man. These Four Elements were Water, Air, Fire and Earth (Schöner 1964). The most famous physicians were Hippocrates (around 400 BC) and Galenus, living in the 2nd century after Christ. In medieval time the system of pathology and therapy, originating from Galenus, dominated Western medicine. The fundamental principle in the so-called Galenism was the transformation of humoral pathology into a rigid dogma (Siegel 1968). Already the school of Hippocrates had formulated the theory of the Four Humours (paralleling the Four Elements), the correct balance of which meant health, while every disturbance of this balance caused disease. There were Four Humours: Blood (coming from the heart), Phlegm (supposed to come from the brain), Yellow Bile (supposed to be secreted from the liver), and Black Bile (supposed to come from the spleen and the stomach). Each of these humours had definite qualities. Blood was moist and warm; Phlegm moist and cold; Yellow Bile warm and dry; Black Bile cold and dry. Furthermore, there was a definite connection between predominance of one humour in the metabolic system and an individual's temperament such as sanguine, phlegmatic, choleric, and melancholic.

European medicine has been influenced by Arabian medicine, mainly by the physician Ali Ibn Sina, who was known in Europe under the name Avicenna. His books were translated into Latin and his *Canon medicinae* has influenced European medicine for centuries (Gruner 1930).

Saint Hildegard of Bingen, the abbess of a monastery, was one of the most famous physicians and pharmacists in the 12th century (Müller 1982). She wrote many books on human nature and the use of herbal drugs. Theophrastus Bombastus von Hohenheim, called Paracelsus, who lived in the 15th century, was a reformer of European medicine (Temkin 1941). One of his ideas was that in nature a remedy could always be found against every disease. The Latin sentence "*Ubi malum, ibi remedium*" was the core idea of the signature theory that the shape or the color of a plant indicates against which disease the herbal drug can be used.

European medicine has undergone a great change in paradigm. In contrast to traditional Chinese medicine and Ayurveda, traditional European medicine does not exist any more and is only a matter of history of medicine. Even the self-understanding of modern phytotherapy in Europe is completely based on allopathy that means modern Western medicine.

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0.2 Pharmacological approaches of modern medicine

The most important achievements of **modern Western medicine** were made in several areas such as diagnosis, infectious diseases, endocrinology and medicinal chemistry. Cellular pathology was founded by Virchow in the 19th century (Pagel 1906). The inten-

sive use of the microscope in medicine with histological comparison of diseased and normal organs allowed the change from humoral to **cellular pathology**.

Medicinal chemistry as an important science started less than 100 years ago. The active principles of plants, mostly alkaloids, were isolated and were the starting point for syntheses, such as:

Morphine and papaverine from *Papaver somniferum* for synthetic analgesics and spasmolytics; atropine from *Atropa belladonna* for synthetic spasmolytics; cocaine from *Erythroxylon coca* for synthetic local anaesthetics; quinine and quinidine from *Cinchona succirubra* for synthetic anti-malaria drugs and antiarrhythmics; ephedrine from *Ephedra sinica* for synthetic sysmpathicomimetics and sysmpathicolytics including β-blockers, xanthines like coffeine; theobromine and theophylline from *Coffea arabica*, *Theobroma cacao*, *Camellia sinensis* for vasotherapeutics such as pentoxyphylline; ergot alkaloids from *Claviceps purpurea* for semisynthetic ergot derivatives; reserpine and ajmaline from *Rauwolfia serpentina* for synthetic antihypertensives and antiarrhythmics; physostigmine from *Physostigma venenosum* for potential antidementic drugs; glycosides from *Digitalis lanata* and *Digitalis purpurea* for semisynthetic cardiac glycosides; anthraquinones from *Senna angustifolia* or *Rhamnus frangula* or *Rheum officinale* for synthetic laxatives.

Pharmacological research started in Europe in the second half of the 19th century when their founders, e.g., Rudolf Buchheim and Oswald Schmiedeberg, investigated the action of existing drugs in animal experiments (Kochweser and Schechter 1978).

With the emergence of synthetic chemistry the pharmacological evaluation of these products for therapeutic indications became necessary. Many new drugs were discovered by this classical approach during the 20th century.

The **classical way of pharmacological screening** involves sequential testing of new chemical entities or extracts from biological material in **isolated organs** followed by **tests in whole animals**, mostly rats and mice but also higher animals if indicated. Most drugs in use nowadays in therapy have been found and evaluated with these methods.

The chemistry and pharmacology of drugs, which have been found and used in therapy until 1970, are described in the 5 volumes of the book: Ehrhart G, Ruschig H (eds) Arzneimittel. Entwicklung, Wirkung Darstellung (1972).

In the mid-1970s **receptor binding assays** were introduced as an approach for compound evaluation by the development of radioligand binding assays, based on evaluation procedures and mathematical calculations provided by Schild (1947), Scatchard (1949), Stephenson (1956), Ariëns and van Rossum (1957), Arunlakshana and Schild (1959), Furchgott (1966), Cheng and Prusoff (1973), Rodbard and Frazier (1975), Bennett (1978), Creese (1978), Munson and Rodbard (1980), McPherson (1985a,b), Tallarida and Murray (1987), Greenstein (1991). Receptor binding assays were described for various transmitters as well as assays for ion channels and neurotransmitter reporters (e.g., by Snyder et al. 1975; Bylund and Snyder 1976; Enna 1978; U'Prichard et al. 1978, 1979; Bruns et al. 1980; Starke 1987; Dohlman et al. 1991; Krogsgaard-Larsen et al. 1991; Snider et al. 1991; Betz 1992; Wisden and Seeburg 1992; Amara and Arriza 1993; Bowery 1993; Wess 1993; Isom et al. 1994; Köhr et al. 1994; Goto and Yamada 1998; Chittajallu et al. 1999; Bormann 2000).

Characterization and classification of receptors is a continuous procedure (William 1991; Kebabian and Neumeyer 1994; Angeli and Guilini 1996; Trist et al. 1997; Godfraind and Vanhoutte 1998; Watling 1998; Alexander et al. 2001).

The use of radioligand binding assays has facilitated the design of new chemical entities, especially as the information obtained has been used in deriving molecular models for the structure-activity relationship. The receptor technology provides a rapid means to evaluate small amounts of compound (5–10 mg) directly for their ability to interact with a receptor or enzyme, independent of its efficacy. But as new assays were developed, it also provides a means to profile the activity of compounds against a battery of binding sites, thereby yielding an *in vitro* radioligand binding profile (Shaw 1992). Receptors have been divided in more and more subclasses (Vanhoutte et al. 1996) so that full characterization of a new chemical entity by receptor pharmacology also needs time and material.

Many receptors have been identified by DNA sequencing technology, mostly belonging to the G-protein coupled receptor superfamily, for which ligands have not yet been identified. Reverse molecular pharmacology and functional genomic strategies are recommended to identify the activating ligands of these receptors (Wilson et al. 1998). The reverse molecular pharmacological methodology includes cloning and expression of G-protein coupled receptors in mammalian cells and screening these cells for a functional response to cognate or surrogate agonists present in biological extract preparations, peptide libraries, and complex compound preparations.

The ligand binding assay is a powerful tool in the search for agonists and antagonists for novel receptors, and for identification of novel classes of agonists and antagonists for known receptors. However, it does not

differentiate between agonist and antagonist. Ligand binding mass screening can be adapted for very high throughput. However, well-defined criteria have to be fulfilled to avoid blind alleys (Burch 1991).

Pharmacological methods, including receptor pharmacology which led to the discovery and evaluation of drugs used in therapy until 2000, are described in the following chapters:

Chapter A Cardiovascular activity
Chapter B Activity on blood constituents
Chapter C Activity on urinary tract
Chapter D Respiratory activity
Chapter E Psychotropic and neurotropic activity
Chapter F Drug effects on learning and memory
Chapter G Effects on peripheral nerve function
Chapter H Analgesic, anti-inflammatory, and anti-pyretic activity
Chapter I Antiarthrotic and immunmodulatory activity
Chapter J Activity on the gastrointestinal tract
Chapter K Antidiabetic activity
Chapter L Anti-obesity activity
Chapter M Anti-atherosclerotic activity
Chapter N Endocrinology
Chapter O Ophthalmologic activity

The **classical approach** has the advantage of relatively high relevance. If a compound has blood pressure lowering activity in hypertensive rats after oral dosage, the chances of activity in humans are high. Measurement of dose-response-curves, effects over a given period of time and comparison of the effects after intravenous and oral administration already give hints for pharmacokinetic data. This approach has the disadvantage that it is time consuming and requires relatively large amounts of the new compound (usually about 5 g). Furthermore, this approach provides little information about the molecular mechanisms involved in the observed effects. On the other hand, one has to keep in mind that millions of diabetics were treated successfully with sulfonylureas over decades whereby only the release of insulin from the pancreatic β-cell, but not the molecular mechanism, was known (Bänder et al. 1969).

One has to admit that not all breakthroughs were achieved by the classical way of drug research. Several drugs have been identified by **serendipity** in the clinic, such as the diuretic action of sulfanilamide and acetazolamide as inhibitors of carbonahydrase (Schwartz 1949; Maren 1960, 1967), the blood sugar-lowering activity of sulfonylureas designed as antibacterial agents (Loubatières 1946; Franke and Fuchs 1955; Achelis and Hardebeck 1955), the antipsychotic of phenothiazines (Delay and Deniker 1952; Laborit et al. 1952; Courvoisier 1956), the antidepressant action of isoniazid (Kline 1958) and of imipramine (Kuhn 1958), the anticonvulsant effects of sodium valproate, or the effect of sildenafil, originally designed as a therapy for angina (Enna 2000).

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0.3 New approaches in drug discovery

0.3.1 Combinatorial chemistry

It was always due to the ingenuity of the chemist that new chemical entities in pharmaceutical research were created (Schapira et al. 2000; Schreiber 2000). For some period of time, computer-based molecular modeling was thought to be the most effective approach. With the increasing characterization of the three-dimensional structures of receptors and enzymes, the design of molecules that interact with these biological targets was thought to be an intellectual approach using modern computer technology (Stigers et al. 1999).

This attitude to the development of **combinatorial chemistry** has changed. It has been defined as the systematic and repetitive covalent connection of a set of different 'building blocks' of varying structures to each other to yield a large array of diverse molecular entities (Gallop et al. 1994; Gordon et al. 1994; Fecik et al. 1998). As chemists gained experience in this technique, it has become possible to use solid- or solution-phase syntheses with different chemistries and scaffolds to produce libraries tailor-made for finding or optimizing a lead directed at almost any class of target (Hogan 1996; Maclean et al. 1997; Appleton 1999; Dooley and Houghten 1999; Lukas et al. 1999; Houghten 2000; Lazo and Wipf 2000; Rademann and Jung 2000). New tools, such as molecular docking algorithms (Burkhard et al. 1999), mapping of protein binding sites by nuclear magnetic resonance (Shuker et al. 1996), preparation of highly enantioselective selectors for chiral HPLC (Lewandowski et al. 1999), light-directed synthesis (LeProust et al. 2000), and homology modelling of proteins (Kiyama et al. 1999), allow an unprecedented level of rational design to guide the synthesis of prospective drugs. Spectroscopic methods, including especially mass spectrometry and to a lesser extend infrared and nuclear magnetic resonance, have been applied at different levels of combinatorial library synthesis: in the rehearsal phase to optimize the chemistry prior to library generation, to confirm library composition, and to characterize after screening each structure that exhibits positive response (Dancík et al. 1999; Hajduk et al. 1999; Loo et al. 1999; Scherer et al. 1999; Cancilla et al. 2000; Enjalbal et al. 2000; Kyranos et al. 2001).

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0.3.2
High throughput screening, ultra high throughput screening, and high content screening

The logic development of receptor technology was high throughput screening. This evolution was closely connected with the changes in strategy of chemical synthesis. The vast number of compounds produced by combinatorial chemistry and the possibility to test many compounds, including natural products (Harvey 2000), in a short period of time by high throughput screening stimulated each other.

With the introduction of robotics, automation and miniaturization techniques, it became feasible to screen 50 000 compounds a day with complex workstations. Full automation of all assay steps, from compound addition to data collection, ultimately allows screens to be run continuously – 24 hours a day, 7 days a week. Another progress is the development of 1 536 well microtiter plate formats (Berg et al. 2000; Dunn et al. 2000). Multiple types of formats are emerging, besides 96-, 384-, and 1 536-, even 3 456- and 9 600-well plates (Sills 1998).

Harding et al. (1997) described the development of an automated high-throughput screening system involving two systems, the first handling isotopic assay ('hot' system), the second non-isotopic assays ('cold' system).

High-content screening (HCS) was proposed as a new approach to easing key bottlenecks in the drug discovery process by Giuliano et al. (1997). HCS defines the role of targets in cell function by combining fluorescence-based reagents with the ArrayScan™ to automatically extract temporal and spatial information about target activities within cells. High content screening integrates cell based assays, high resolution fluorescence microscopy, and proprietary image processing algorithms for the automated analysis of cellular and subcellular events. HCS delivers the potential to screen using targets that were minimally used or avoided due to a lack of a robust way to measure them, such as morphology changes, cellular differentiation and cytoskeletal changes; cell to cell interactions, chemotaxis and motility, spatial distribution changes like receptor trafficking or complex formation.

Clark and Pickett (2000) underlined the importance of computational methods for the prediction of 'drug-likeness', in particular for the prediction of intestinal absorption and blood-brain barrier penetration.

Several authors expressed their thoughts on the future of high throughput screening (Stahl 1999; Divers 1999; Eglen 1999; Fox et al. 1999, 2001; Mander 2000). Valler and Green (2000) expressed some doubts on diversity screening in drug discovery and proposed focussed screening using three-dimensional information of the target which may improve the hit rate 10–100 fold over random screening.

Nevertheless, this technical progress has led to a new concept in drug discovery: "a change in paradigm". Large numbers of hypothetical targets are incorporated into *in vitro* or cell-based assays and are exposed to large numbers of compounds of natural or synthetic origin.

This change in paradigm created new areas, such as bioinformatics, cheminformatics, and functional genomics (Ohlstein et al. 2000; Ryu and Nam 2000).

Many compounds can elicit a positive response in a particular assay, which are called hits. In this new terminology **"Hit"** is a molecule with confirmed activity from the primary high throughput-screening assay, a good profile in secondary assays and a confirmed structure.

The hits have to be structurally defined and may give rise to leads (Lahana 1999). **"Lead"** is a hit series for which the structure-activity relationship is shown and activity demonstrated both *in vitro* and *in vivo*.

The implementation of these methods is being complemented by an increase in the use of automation and robotics. Automation may be defined as the use of stand-alone instrumentation (work stations) that perform a given task whereas robotics use a robot arm or track systems to move microplates between these instruments. These technologies are continuously improved (Brown and Proulx 1997; Lightbody and Alderman 2001; Ruch 2001).

High throughput screening methods are also used to characterize metabolic and pharmacokinetic data of new drugs (Watt et al. 2000; White 2000).

A large pharmaceutical company has the potential to screen up to 100 000 000 compounds per year (Drews 2000). In any case, the leads or the derivatives from them have to be tested in more complex models, isolated organs or animals. Only compounds active in classical pharmacological tests can be taken into preclinical and clinical development.

With the completion of the human genome sequencing, bioinformatics will be an essential tool in target discovery and the *in silico* analysis of gene expression and gene function will be an integral part of it, facilitating the selection of the most important targets for a disease under study (Terstappen and Reggiani 2001).

Some doubts were expressed on the philosophy of research management in pharmaceutical industry neglecting the influence of creative scientists, failure to understand biological complexity and overestimating the outcome of the "change in paradigm" (Horrobin 2000). Nevertheless, the race to test more and more compounds in a shorter time in ultra high throughput screening with more automation is continuing.

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0.3.3 Technologies for high throughput screening

Fundamental changes in assay technologies have facilitated the development of high throughput screening (Major 1995; Pazhanisamy et al. 1995; Devlin 1997; Harding et al. 1997; Houston and Banks 1997; Sittampalam et al. 1997; Su et al. 1997; Fernandes 1998; Gonzalez and Negulescu 1998; Kenny et al. 1998; Pasini et al. 1998; Silverman et al. 1998; Zysk and Baumbach 1998; Brandt 1998; Sills 1998; Wingfield 1998; Bolger 1999; Labute 1999; Winkler et al. 1999; Bosse et al. 2000; Cox et al. 2000; Feiglin et al. 2000; Gauglitz 2000; Landro et al. 2000; Meza 2000; Parker et al. 2000; Schuster et al. 2000; Sundberg 2000).

Dispensing technologies

Several systems for fluid dispensing are in use: The piezo- and inkjet, the "air displacement", and the "pintool" systems (Vollert et al. 2000). Piezoelectric and inkjet systems were the first introduced to the market (Lemmo et al. 1998; Rose 1999). There are three types of inkjet dispensers: thermal, solenoid and piezoelectric. All of these use some means of compressing a liquid against a small orifice to create sufficient linear velocity to eject the fluid in the form of a drop.

The "pintool" systems use pins (needles) to transfer liquid. Up to 1 536 needles are immersed into the fluid and withdrawn, taking up a few nanoliter and transferring the fluid into the wells of a microtiter plate.

Utilization of syringes and syringe needles as a contact tool enables multiple dispensing from a single fill. Generally, syringe dispensing has a higher degree of accuracy and repeatability compared with pin dispensing tools (Vollert 1998; Dunn and Feygin 2000).

Microfluidic arrays for high throughput submicroliter assays using capillary electrophoresis were described by Gibbons (2000). Capillary electrophoresis is an analytical technique that can achieve rapid high-resolution separation of water-soluble components present in small sample volumes. Separations are based on the electrically driven movement of ions. In high throughput screening, analytes are separated according to differences in electrophoretic mobility, depending on the charge-to-size ratio.

Cell-based assays

Cell-based assays are increasingly attractive alternatives to *in vitro* biochemical assays for HTS (Wallace and Goldman 1997; Silverman et al. 1998). They fall into three broad categories: second messenger assays that monitor signal transduction following activation of cell-surface receptors; reporter gene assays that monitor cellular responses at the transcription/translation level; and cell proliferation assays to monitor the overall growth/no growth response of cells to external stimuli (Sundberg 2000).

Cell-based reporter assays are used where human receptors are transfected into null cell lines either alone, or as part of receptor systems constructed to show alterations in light production (luciferin-luciferase) or light transmission (melanophore), that can be measured independently of radioactivity within minutes (Broach and Thorner 1996).

Pathirna et al. (1995) developed a transient transfection HTS assay to identify nonsteroidal human progesterone receptor modulators using luciferase expression (see also N.3.2.1.2).

Another reporter gene, green fluorescent protein, can be measured by fluorescence techniques in real time so that kinetic parameters can be determined from a single well of a microtiter plate (Chalfie et al. 1994; Chalfie 1995; Kain 1999; Meng et al. 2000).

Scheirer (1997) reviewed the various reporter genes and their applications.

González and Negulescu (1998) described intracellular detection assays for high throughput screening which employ either fluorescent or luminescent readouts. The method is particularly suitable to measure calcium mobilization (Akong et al. 1955; Burbaum and Sigal 1997).

Using receptor selection and amplification technology (R-SAT), it is possible to rapidly quantify specific pharmacological responses as a function of changes in cell proliferation rates (Messier et al. 1995). NIH3T3 cells (or other cell lines) are transfected with the plasmid coding for the receptor of interest and pSV-β-Gal. Cells are then transferred to 96-well microtiter plates and test compounds are added. Several days later, β-Gal activity is measured via a simple, inexpensive colorimetric assay. As a result of compound treatment, the changes in enzyme activity are a function of the proliferative activity of the cells. This functional assay can quantitatively differentiate full agonists, partial agonists, neutral antagonists, and negative antagonists (inverse agonists). An interesting aspect of this technology is that many genes in a related family can be combined in the same cell population for convenient assay.

Detection methods

Fluorescence-based assay technologies play an increasing role in high throughput screening. When compared to other biochemical and cell-based techniques, fluorescence has a significant advantage over such methods as isotopic labeling, colorimetry, and chemoluminiscence.

Sensitivity. It is relatively simple for modern instrumentation to reliably detect a signal being emitted from a single fluorescent molecule. In biological applications, this level of sensitivity means molecules that may be present only in very small numbers can be easily detected. In the case of imaging application, such as High Content Screening, their intracellular location can be determined. This high level of sensitivity also means that transient biological events can be detected very quickly, hence enabling the measurement and understanding of events that occur very rapidly inside living cells. The inherent sensitivity of fluorescence technology also permits the use of very low concentrations of fluorescent label.

Specificity. Fluorescent probes are readily available for labeling virtually any biomolecule, structure, or cell type. Immunofluorescent probes can be directed to bind not only to specific proteins but also to specific conformations, cleavage products, or site modifications like phosphorylation. Individual peptides and proteins can be engineered to autofluorescence by expressing them as AFP chimeras inside cells. Such high levels of specificity enable the use of several different fluorescent labels – each emitting at a unique color – and the subsequent understanding of the complex interactions that occur among and between subcellular constituents.

Accuracy. Reagents used for fluorescence labeling are well understood and their performance has been characterized under a wide variety of biological environments.

Flexibility. Fluorescence technologies have matured to the point where an abundance of useful dyes are now commercially available. Many dyes have been developed and optimized for labeling specific cell compartments and components, as well as for labeling proteins (antibodies, ligands, toxins, etc.) which can be used as probes. Other fluorescent sensors have been designed to report on biological activities or environmental changes (pH, calcium concentration, electrical potential, proximity to other probes etc.). Multiple fluorescent labels can be used on the same sample and individually detected quantitatively, permitting measurement of multiple cellular responses simultaneously. Many quantitative techniques have been developed to harness unique properties of fluorescence, including:

- Fluorescence resonance energy transfer (FRET),
- Fluorescence polarization or anisotropy (FP),
- Time resolved fluorescence (TRF),
- Fluorescence lifetime measurements (FLM),
- Fluorescence correlation spectroscopy (FCS),
- Fluorescence photobleaching recovery (FPR).

Mere et al. (1999) reported miniaturized **fluorescence resonance energy transfer (FRET)** assays and micro-fluidics as key components for ultra-high-throughput screening. An ultra-high throughput screening system (UHTSS™) platform integrates several microfluidic dispensing devices, which have been developed to deliver nanoliter to microliter volumes at high speed into the appropriate wells of 384-well NanoWell™ assay plates. The activity of the compounds dispensed into the assay wells is quantified via a highly sensitive dual-wavelength emission fluorescence detector.

Pope et al. (1999) discussed the advantages and disadvantages/limitations of fluorescence techniques for the establishment of miniaturized homogeneous screening assays, including prompt intensity assay (FLINT), anisotropy assay (FA), prompt energy transfer assay (FRET), fluorescence correlation spectroscopy (mass-dependent FCS), and fluorescent correlation spectroscopy (mass-independent FCS).

Auer et al. (1999) recommended fluorescence correlation spectroscopy for lead discovery by miniaturized HTS. By monitoring interactions of single molecules in fentoliter volumes, fluorescence correlation spectroscopy offers the highest potential as the detection technique in the nanoscale.

Haupts et al. (2000) compared macroscopic versus microscopic fluorescence techniques in (ultra)-high throughput screening.

Sportsman and Leytes (2000) discussed miniaturization of homogenous assays using **fluorescence polarization (FP)**. Fluorescence polarization can detect changes in molecule size as well as quantify the binding of small molecules to larger molecules. Polarization of fluorescence occurs when a fluorescent molecule is illuminated with plane-polarized light, provided that the molecule does not move during the course of fluorescent lifetime. For typical fluorescent molecules such as fluorescein, the polarization is not observed if the molecule is rotating rapidly in solution under condition typically used in biological assays. Thus, if the polarization of fluorescence of a fluorescein-labeled ligand is measured, a low polarization will be observed when the ligand is free in solution, and a high polarization will be observed when the ligand is bound to a macromolecule such as a specific receptor or antibody.

Banks et al. (2000) described fluorescence polarization assays for high throughput screening of G protein-coupled receptors in 384-well microtiter plates.

Parker et al. (2000) used fluorescence polarization to develop high throughput screening assays for nuclear receptor-displacement and kinase inhibition. This method is a solution-based, homogeneous technique, requiring no immobilization or separation of reaction components. As an example, the authors described the fluorescence polarization-based estrogen receptor assay based on the competition of fluorescein-labeled estradiol and estrogen-like compounds for binding to the estrogen receptor.

Li et al. (2000) developed an assay for measuring the activity of an enzyme that transfers multiple adenine-containing groups to an acceptor protein, based on fluorescence polarization technology in a 1 536-well plate format. Texas red (rhodamine) was covalently conjugated to adenine of the donor substrate through a C_6 spacer arm. As a result of the transfer of the adenine-containing moieties to the acceptor protein substrate, the rotational correlation time of the Texas red conjugate increased, hence increasing the degree of fluorescence polarization.

Kolb et al. (1997), Grepin and Pernelle (2000) described evolution of **homogeneous time resolved fluorescence (HTRF)** technology for HTS. HTRF uses Eu^{3+} ion caged into a polycyclic cryptate (Eu-cryptate) as donor. Laser excitation at 337 nm transfers the energy from the Eu-cryptate complex to an allophycocyanin acceptor molecule (XL665), the APC. This results in the emission of light at 665 nm over a prolonged time (milliseconds). Uncoupled or free XL665 emits at 665 nm, but with a short decay. The light emission is recorded in a time-resolved fashion over a 400 µs period, starting 50 µs after the excitation pulse, so that the autofluorescence from the media and the

short-lived fluorescence of the free APC are not recorded. Eu-cryptate emits at 620 nm and is discriminated from XL665 by wavelength.

The labeling of macromolecules for HTRF assay can be done in a number of ways, depending on the type of screen, using direct, indirect or semi-direct types of labeling. Direct labeling is characterized by having the (Eu)K and XL665 covalently bound to the molecule or molecules of interest. Indirect labeling is when the (Eu)K and XL665 are bound to macromolecules through secondary interactions, such as biotin-streptavidin interaction or antibody binding. Other indirect methods, such as (Eu)K or XL665 labeled lectin, can be used for binding to membranes. The semi-direct method is a combination of direct and indirect and is frequently used in HTRF.

HTRF immunoassays generally use a direct labeling strategy in which two antibodies against different antigenic sites are used. One antibody is labeled with (Eu)K and another is labeled with XL665. When both bind to the antigen, energy transfer occurs and the long-lived XL665 signal is generated.

To study the interaction of two proteins, the JUN FOS dimerization was used as a model assay. The JUN protein was biotinylated. Streptavidin:(Eu)K was used to bind that label to the JUN-biotin protein via the SA-biotin binding. The FOS peptide was directly labeled with XL665. Signal is only generated when JUN-biotin:SA-(Eu)K and FOS-XL665 dimerize to form the activated AP1 transcription complex.

The binding of ligands to membrane receptors and the competitive displacement by unknown molecules is one of the most common screening assays in drug discovery. To test the applicability of HTRF for receptor binding, the interaction of epidermal growth factor (EGF) with its receptor (EGFR) was studied (Mathis et al. 1994). To generate a HTRF signal, a semidirect labeling method was developed. EGF was labeled directly with (Eu)K, and a monoclonal antibody against a non-binding portion of the EGF receptor was used. The anti-EGRF antibody was labeled with XL665. The IC_{50} of displacement could be determined.

The phosphorylation of enzymes, proteins and receptors is a major mechanism of cell regulatory pathways and is therefore a frequent target for drug discovery. The epidermal growth factor receptor-intrinsic tyrosine kinase activity was used to develop an HTRF assay. Biotin labeled $(glutamine\text{-}alanine\text{-}tyrosine)_n$ (Poly GAT) was used as a substrate. The EGF receptor was isolated from A431 cells after partial purification of a cell homogenate. After the phosphorylation reaction, the HTRF signal was generated by adding streptavidin-XL665 and (Eu)-K-antiphosphotyrosine antibody. To establish the biological function of HTRF in this assay, inhibition of tryphostin-47 was assayed.

The **acumenexplorer** is a laser-scanning system that measures fluorescence and luminescence, combining the ability to detect sub-micron events and features with ultra-fast acquisition and data management. Different parts of the cell can be localized by labeling with Hoechst 33342, Alexa™ 488, Alexa™ 546, Alexa™ 594, or Cys™ 5. Many different colors (readouts) can be used simultaneously.

The **fluorometric imaging plate reader (FLIPR™)** permits rapid, kinetic measurements of intracellular fluorescence. Simultaneous measurements in 96 wells and in real time can be made every second: before, during and after the addition of test compounds. The detection optics of FLIPR are based on cooled charge-coupled device (CCD) technology. With each kinetic update, the system takes a picture of the bottom of a microplate, recording a signal for all the individual cells simultaneously. Enhanced sensitivity for cell-based assays is accomplished via an optical detection scheme, which allows for signal isolation on a cell monolayer. The system is ideal for monitoring intracellular calcium fluxes that occur within seconds of activation of G-coupled receptors. Using calcium sensitive dyes such as Fluo-3 and this permits the derivation of full dose response (or inhibition) curves in a matter of minutes (Kuntzweiler et al. 1998; Coward et al. 1999; Miller et al. 1999; Milligan and Rees 1999; Sullivan et al. 1999).

Swartzman et al. (1999) developed a simple, homogeneous bead-based immunoassay for use with **fluorometric microvolume assay technology (FMAT)**.

Koltermann et al. (1998) proposed rapid assay processing by integration of **dual-color fluorescence cross-correlation spectroscopy (RAPID FCS)** as an ideal tool for ultra high throughput screening when combined with nanotechnology, which can probe up to 10^5 samples per day. Auer et al. (1999) used fluorescence correlation spectroscopy for lead discovery by miniaturized HTS.

Winkler et al. (1999) presented **confocal fluorescence coincidence analysis** as an alternative for high throughput screening. Confocal fluorescence coincidence analysis extracts fluorescence fluctuations that occur coincidentally in two different spectral ranges from a tiny observation volume of below 1 fl. This procedure makes it possible to monitor whether an association between molecular fragments that are labeled with different fluorophores is established or broken, providing access to the characterization of a variety of cleavage and ligation reactions in biochemistry.

For high throughput screening, Schmid et al. (1998) proposed a method to reversibly attach receptor proteins via an affinity tag to a quartz surface and subsequently detect with high sensitivity the real-time binding of ligands by **total internal reflection fluorescence**.

The **CytoFluor® Fluorescence Multi-Well Plate Reader** is a versatile fluorescence intensity measurement instrument that detects and quantifies fluorescent molecules over a five-log dynamic range. The system has a sensitivity better than 8 femtomoles/well for fluorescein.

Ramm (1999) described the technique of **image-based screening in the LEADseeker™ system**, which contains a cooled charge-coupled device (CCD) camera and a telecentric lens with integral epifluorescence illumination.

Laser scanning imaging systems have been developed to measure cellular and subcellular quantitation of fluorescence in whole cells (Conway et al. 1999; Zuck et al. 1999; Hertzberg and Pope 2000).

Chemiluminescence is a photometric technique that is applicable to HTS. Detection of chemiluminescence is a convenient adjunct to fluorescence, since most plate readers capable of measuring fluorescence will measure luminescence as well. This technique has been used predominantly with luciferase reporter genes in cell-based assays and in high sensitivity enzyme-linked immunosorbent assays (ELISA), employing chemolumogenic substrates for alkaline phosphatase and horseradish peroxidase (Bronstein et al. 1994, 1996). The Luc-Screen® assay system with extended-glow light emission was designed for sensitive detection of firefly luciferase reporter enzyme. Liochev and Fridovich (1997) recommended lucigenin luminescence as a measure of intracellular superoxide dismutase activity in *Escherichia coli*.

The NorthStar™ HTS workstation has been developed as a chemiluminescence analyzer for the high throughput screening laboratory. Featuring online injection, a broad dynamic range, and an extremely low crosstalk, the NorthStar™ HTS workstation allows the routine analysis of more than 500 000 samples a day.

Grépin et al. (2001) presented an assay for precise and direct quantification of specific endogenous mRNAs in cell lysates. The technology is based on the bioluminescence detection of multiple biotinylated ssDNA probe/endogenous mRNA hybrids, which are captured onto a streptavidine coated multi-well plate. Using the Xpress-Screen™ technology, an assay was developed aimed to monitor the induction of endogenous cFos mRNA expression in NGF-treated PC12 cells and the miniaturization of the assay in 384 well format, which is adapted to high throughput screening.

The introduction of the **scintillation proximity assay (SPA)** obviated the need to separate bound from free radioactivity in the conventional radioligand-binding assay (Bosworth and Towers 1989; Picardo and Hughes 1997; Game et al. 1998). One version of the scintillation proximity assay utilized polyvinyltoluene microspheres or beads into which a scillitant has been incorporated. When a radiolabeled ligand is captured on the surface of the bead, the radioactive decay occurs in close proximity to the bead, and effectively transfers energy to the scillitant, which results in light emission (Sittampalam et al. 1997).

FlashPlate™ technology has been described by Brown et al. (1997). FlashPlates are white 96-well polystyrene microplates coated on the inside with solid scillitant in a polystyrene polymer. The polystyrene surface provides a hydrophobic surface for absorption of proteins, such as antibodies and receptors. When the binder molecule is coated on the FlashPlate, the reaction of the mixture of radiolabeled tracer and unlabeled ligand of interest with the binder molecule, and detection of the bound radioactivity can be done in one step. Due to the microplate surface scintillation effect, only the bound radioactivity will be detected by a scintillation counter.

The use of **high-performance microphysiometry** in drug discovery has been discussed by Alajoki et al. (1997). Microphysiometry measures the extracellular acidification rate. It is governed by catabolism, via the excretion of acidic end-products, such as carbon dioxide and lactic acid. In addition, it is substantially influenced in a complementary fashion by the regulation of intracellular pH, a process sensitive to physiological changes such as receptor activation (see also A.1.1.7 and E.5.1.2). The Light-Addressable Potentiometric Sensor (LAPS) uses a light-generated alternating current to probe the electrical potential at the interface between an electrolyte and the insulated surface of an appropriately biased silicon chip; the surface potential depends on pH in a Nernstian fashion. This system has been optimized for high throughput screening.

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0.3.4 Pharmacogenomics

The concept of an altered response based on genetic background is not new. In 510 BC, Pythagoras recognized that some individuals developed hemolytic anemia with fava bean consumption (Nebert 1999). In 1914, Garrod expanded this observation, stating that enzymes detoxified foreign agents so that they were excreted harmlessly. However, some people lack these enzymes and experience adverse effects (Weber 1999). Hemolytic anemia due to fava bean consumption was later determined to occur in glucose-6-phosphate deficient individuals (Mager et al. 1965; Podda et al. 1969).

The term pharmacogenetics was first used by Friedrich Vogel in 1959. The dawn of pharmacogenetics continued by combining Mendellian genetics with observed phenotypes. In 1932, Snyder performed the first global study of ethnic variation and deduced that taste deficiency was inherited. He proposed that the phenylthiourea non-taster phenotype was an inherited

recessive trait and that the frequency of occurrence differed between races (Snyder 1932). Other genetic differences, such as aldehyde dehydrogenase and alcohol dehydrogenase, were discovered. These deficiencies are common in Asians and cause them to have less alcohol tolerance (Goedde et al. 1983; Inoue et al. 1984). Similarly, polymorphisms in the *N*-acetyl transferase enzyme are also segregated by ethnicity and correlate to the latitude of the country (Weber 1999).

These genetic differences were originally thought to be caused by genetic variance, but it was not until the advent of molecular biology that disease states could be carefully analyzed. The following dogma:

gene → protein → biochemical process → disease state

became the model for examining human diseases. Following this scheme, sickle cell anemia was the first trait to reveal that a single point mutation can change protein structure and lead to a disease phenotype (Murayama 1966; Bookchin et al. 1970). Single nucleotide polymorphisms (SNPs) are responsible for many diseases (Kleyn and Vesell 1998; Vesell 2000; Weaver 2001; Wieczorek and Tsongalis 2001). SNPs can change receptors, transport proteins, and drug metabolizing enzymes. The advances in genetic technology to detect polymorphisms have caused an explosion in pharmacogenetic research and many of these discoveries have been employed into clinical practice.

Many thousands of polymorphisms can be determined simultaneously in a patient. These single nucleotide polymorphisms are selected as markers evenly distributed to the genome, in the hope that functionally relevant polymorphisms can be associated with specific markers by virtue of their proximity to the chromosome. Such genome-wide association studies are used in the discovery of susceptibility genes for diseases, such as asthma and prostate cancer, but they are equally suitable for determining the genes in drug response (Sadée 1999).

Disorders like Huntington's disease or cystic fibrosis are associated with defects in a single gene product identified by positional cloning. This involves the isolation of a gene with the information on its subchromosomal localization and the phenotypic expression of a mutation of this gene that results in a particular disease state. Positional cloning is based on the unique, inheritable DNA fingerprint associated with each individual, from which the inheritance of identified polymorphisms can be tracked, along with the inheritance of the disease. While this approach has been more or less successful in diseases involving a single gene defect, more complex diseases, like Alzheimer's disease, cancer, asthma or cardiovascular diseases, appear to result from the influence of multiple gene defects.

The next step is to identify the function of the protein expressed by the DNA using functional genomics. This technique involves the use of sequence comparisons with known proteins, using computer databases, transgenic expression or expression in surrogate systems; gene knockouts in mice (Rudolph and Möhler 1999; West et al. 2000), or simpler organisms like *Drosophila melanogaster*, *Caenorhabditis elegans* (Link et al. 2000) or *Saccharomyces cerevisiae*. A two-hybrid system to identify molecular binding partners can be used to identify proteins that are associated with the gene product of interest.

Progress in genomics technology, especially the elucidation of the human genome (International Genome Sequencing Consortium 2001; Venter et al. 2001), has created an unique opportunity to significantly impact the pharmaceutical drug development processes (Carulli et al. 1998; van Oosterhout 1998; Debouck and Goodfellow 1999; Farber 1999; Vidal and Endoh 1999; Wilson et al. 1998; Jones and Fitzpatrick 1999; Zweiger 1999; Beeley et al. 2000; Bentley 2000; Broder and Venter 2000; Debouck and Metcalf 2000; Harris 2000; Marshall 2000; Meldrum 2000a,b; Rockett and Dix 2000; Schuster et al. 2000; Steiner and Anderson 2000; Peet and Bey 2001; Winkelmann 2001).

Genetic polymorphism in drug-metabolizing enzymes, transporters, receptors, and other drug targets are linked to individual differences in the efficacy and toxicity of many medications. Pharmacogenomic studies can elucidate the inherited nature of these differences, thereby enhancing the possibilities of drug discovery and providing a stronger scientific basis for optimizing drug therapy on the basis of each patient's genetic constitution (Evans and Relling 1999; Grant 2001).

Receptor polymorphism is also the reason for differences in drug effects among patients. Two genetic variants of the angiotensin-converting enzyme are described, depending on an insertion (I-form) or deletion (D-form) of a base pair at position 287 in the gene, which are equally distributed in the Caucasian population. Individuals with the D/D-form express ACE levels much higher than I/I individuals. They have an increased risk for myocardial infarction and may respond better to therapy with ACE inhibitors (Samani et al. 1996; Danser and Schunkert 2000). Another example is the association between β_2-adrenergic receptor polymorphisms and asthma (Liggett 1997). There are three polymorphisms that alter receptor function, which may influence the therapeutic success.

Genomics will introduce a new dimension in drug research. The number of molecular targets that are modified by the complete armamentarium of modern drugs is not greater than 500. The number of genes that contribute to multifactorial diseases might not be very high; the numbers reported for different forms of

diabetes and hypertension are 5–10 per disease (Guillausseau et al. 1997; Shimkets and Lifton 1996). There are 100–150 disease entities that present an epidemiological and economical problem to industrialized societies. If one assumes 10 contributing genes for 100 multifactorial diseases (including different forms of cancer, asthma, diabetes, hypertension, atherosclerosis and osteoporosis), one arrives at 1 000 'disease genes' that dispose patients to the most important multigenetic conditions (Drews 2000, 2001). It appears reasonable to assume that each of these disease genes, or rather proteins that are specified by the disease genes, connects with at least 5–10 proteins that are feasible levels for drug intervention. On the basis of these calculations, one can assume that 5 000 to 10 000 can be used as targets for drug interventions.

Lennon (2000) discussed the methods for high throughput gene expression analysis applicable for drug discovery, including differential display of eukaryotic mRNA, the modification thereof, called Restriction Enzyme Analysis of Differentially Expressed Sequences (READS), expression sequence tags (EST) methodology, serial analysis of gene expression, filter arrays, and DNA microarrays.

Celis et al. (2000) gave an excellent survey on microarray technology.

Gene expression analyses and genetic polymorphisms are not only important for determining predisposition to disease and for drug discovery, but also for predicting the incidence of adverse drug reactions (Meyer 2000). In the past, several drugs had to be withdrawn from the market due to rare, but severe, adverse events. Some drug metabolic enzyme variants have been found to cause severe adverse events. They form qualitatively or quantitatively different metabolites with toxicological implications (Bullingham 2001).

Most of the enzymes involved in drug metabolism and the elimination of many therapeutic agents are members of the cytochrome P450 (CYP) family which includes more than 30 isoforms (Gonzales 1990). One drug metabolizing enzyme, resulting in numerous drugs being withdrawn from the market, is CYP3A, which is involved in the oxidative biotransformation of up to 50% of clinically important therapeutic agents. The expression of CYP3A is regulated by genetic and non-genetic factors that can result in a 5–20-fold interindividual variability in metabolic clearance. Another enzyme, CYP2D6 (debrisoquine hydroxylase), metabolizes one quarter of all prescribed drugs and is inactive in 6% of the Caucasian population (Wolf and Smith 1999). A principal molecular defect in poor metabolizers is a single base pair mutation in exon 5 of CYP2C19 (De Morais et al. 1994; Sadée 1999). These enzymes are being screened at the earliest stages of the drug development process. The single nucleotide point mutation profile of an individual can indicate a predisposition for adverse side effects. Furthermore, drug transport protein polymorphisms and their drug interactions may provide another valuable tool in screening for potential toxic effects of drugs. The pharmacogenomic approach should reduce the number of compounds failing during late clinical development (Adam et al. 2000).

A detailed knowledge of the genetic basis of individual drug response is of major clinical and economic importance and can provide the basis for a rational approach to drug prescription. In the sense of a 'personalized medicine', prospective genotyping will lead to patients being prescribed drugs which are both safer and more effective (Sadée 1999; March 2000; Murphy 2000; Spear et al. 2001).

Winkelmann et al. (2001) initiated the Ludwigshafen Risk and Cardiovascular Health (LURIC) study, which aims to provide a resource for the study of environmental and genetic risk factors and their interactions, and of functional relationship between gene variation and biochemical phenotype (functional genomics), or response to medication (pharmacogenomics) and long-term prognosis of cardiovascular disease.

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0.3.5 Proteomics

The term "proteome" was coined by Wilkins et al. (1995) to describe the protein complement of the genome. The term proteomics was first used to describe the two-dimensional gel electrophoresis (2-DE) and quantitative image analysis. 2-DE remains the highest resolution protein separation method available, but the ability to identify the observed proteins was always an extremely difficult problem. Mass spectrometry (MS) has been an integral part to solving this problem (Fields 2001; Mann et al. 2001). Although improvements in 2-D gel technology had been realized since its introduction, three enabling technological advances have provided the basis for the foundation of the field of proteomics (Patterson 2000). The first advance was the introduction of large-scale nucleotide sequencing of both expressed sequence tags (ESTs) and genomic DNA. The second was the development of mass spectrometers able to ionize and mass-analyze biological molecules and the widespread introduction of mass spectrometers, capable of data-dependent ion selection for fragmentation (MS/MS) (i.e., without the need for user intervention). The third was the development of computer algorithms able to match uninterpreted (or partially interpreted) MS/MS spectra with translations of the nucleotide sequence databases, thereby tying together the first technological advances. The mass spectrometer instruments are named for their type of ionization source and mass analyzer (Patterson and Abersold 1995; Carr and Annan 1997; Patterson 1998). To measure the mass of molecules, the test material must be charged (ionized) and desolvated. The two most successful mechanisms of ionization of peptides and proteins are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). In MALDI the analyte of interest is embedded in a matrix that is dried and then volatilized in a vacuum under ultraviolet laser irradiation. This is a relatively efficient process that ablates only a small portion of the analyte with each laser shot. Typically, the mass analyzer coupled with MALDI is a time-of-flight (TOF) mass analyzer, that measures the elapsed time from acceleration of the charged (ionized) molecules through a field-free drift region (Kowalski and Stoerker 2000). The other common ionization source is ESI, in which the analyte is sprayed from a fine needle at high voltage toward the inlet of the mass spectrometer (which is under vacuum) at a lower voltage. The spray is typically either from a reversed-phase HPLC column or a nanospray device (Wilm and Mann 1994), similar to a microinjection needle. The ions formed during this process are directed into the mass analyzer, which could be a triple-quadrupole, an ion trap, a Fourier-transform ion cyclotron resonance (FT-ICR), or a hybrid quadrupole TOF (Qp-TOF) type (Morris et al. 1996). Although unambiguous identification of a protein cannot always be derived from the masses of a few of its peptides in the tandem mass spectrometer, peptide ions from the first mass spectrometer run are fragmented and identified in a second run to yield a more valuable commodity of a peptide sequence.

The goal of proteomics is a comprehensive, quantitative description of protein expression and its change under the influence of biological perturbations, such as disease and drug treatment (Anderson and Anderson 1998; Müllner et al. 1998; Blackstock and Weir 1999; Dove 1999; Hatzimanikatis et al. 1999; Jungblut et al. 1999; Williams 1999). A combination of mRNA and protein expression patterns has to be simultaneously considered to develop a conceptual understanding of the functional architecture of genomes and gene networks (Kreider 2000). New methods are created like automated proteomics platforms (Quadroni and James 1999; Nielsen et al. 1999), combining two-dimensional electrophoresis, automated spot picking and mass spectrometry (Binz et al. 1999; Dancik et al. 1999; Loo et al. 1999; Dutt and Lee 2000; Feng 2000; Patterson et al. 2000; Ryu and Nam 2000; Service 2000; Yates 2000; James 2001; Jain 2001; Rabilloud 2001).

Improvements in quality, ability and utility of large-scale tertiary and quartery protein structural information are enabling a revolution in rational design, having a particular impact on drug discovery and optimization (Maggio and Ramnarayan 2001).

One may expect that with the new approach of drug research, including combinatorial chemistry, genomics, pharmacogenomics, proteomics, and bioinformatics, unprecedented results will be generated (Burley et al. 1999; Debouck and Metcalf 2000; Drews 2000; Haystead 2001).

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0.3.6 Array technology

Array technology is based on the RNA and DNA hybridization reaction previously used in Northern and Southern expression analysis. However, these traditional approaches allowed only detection of one single gene. In contrast, gene arrays and microarrays allow the analysis of hundreds or thousands of genes simultaneously (DeRisi and Iyer 1999; Diehn et al. 2000; Epstein and Butow 2000; Rockett and Dix 2000; Deyholos et al. 2001; Jordan 2001; Taniguchi et al. 2001). Gene chips can be global, containing the entire genome of one species on the slide. Besides the miniaturization, the greatest leap of technology was the development of fluorescent-labeled nucleotides, which can be detected by laser scanning. They have replaced the more cumbersome and hazardous use of radioactive-labeled DNA.

Two different methods are used in array production: chips are either loaded with synthetic oligonucleotides or with a DNA fragment isolated directly from the respective gene. A specific process allows the synthesis of oligonucleotides directly onto the glass surface. Normally, every gene is represented by about 20 oligonucleotides from different regions of the gene. Each oligonucleotide is 25–30 base pairs in length and

is synthesized next to a control oligonucleotide with a mismatch. The development of DNA chips has been pioneered by researchers at Stanford University (Brown and Botstein 1999). The Stanford Microarray Database has been made widely available (Sherlock et al. 2001) and can be accessed at *http://genome-www.stanford.edu/microarray*.

DNA fragments of 200–400 base pair length are amplified from the gene of interest by PCR and spotted onto poly-lysine or otherwise coated slides (Nature Office 2000). Femto gram amounts of DNA are loaded per spot, which can be less than 50 µm apart from each other.

Regardless of using oligonucleotides or DNA arrays, one always needs two RNA samples for chip analysis. The RNA can be isolated from treated versus untreated cell lines, from the serum of an animal before or after drug administration, or from tumor versus normal tissue. Standard protocols require about 100 µg total RNA, from which poly A^+ RNA is extracted. Next, the RNA is converted by reverse transcription into cDNA in the presence of fluorescent-labeled dyes. Commonly, the sample is labeled with Cy-5 and the control with Cy-3. Both cDNAs are combined and hybridized against the array (Rautenstrauß and Liehr 2001). Since DNA-DNA binding reactions are thermodynamically slow, the chip is incubated overnight at temperature above 60 °C. Chip analysis is performed by scanning with two lasers at 562 nm (Cy-3) and 644 nm (Cy-5). The overlay of both scans shows genes that are deregulated. By convention, overexpressed genes are shown in green, repressed genes in red and unchanged expression in yellow. Data are evaluated by scatter blot analysis, where relative expression of each gene is indicated by logarithmic axes. Sophisticated bioinformatic protocols are used for further annotation and data comparison (Zhang 1999; Gaasterland and Berikanow 2000).

The best approach to microarray analysis is to begin with a small number of the elements in the microarray known to be a pattern and ask questions of the other elements in the microarray; i.e., perform instantaneous scientific experiments regarding whether each of the other elements in the microarray are related to the known pattern (Burke 2000).

Ge (2000) described the development of a universal protein array (UPA) system that provides a sensitive, quantitative, multi-purpose, effective and easy technology to determine not only specific protein-protein interactions, but also specific interactions of proteins with DNA, RNA, ligands and other small chemicals.

The application of biochip and microarray systems in pharmacogenomics has been discussed by Jain (2000).

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0.4 Errors in screening procedures

Any screening procedure has a characteristic error rate. This is inevitable because in high throughput screening, it is especially necessary to sacrifice some accuracy or precision to achieve the requisite speed. Thus when a large number of compounds are carried through a particular screen, some of the compounds will be classified incorrectly. A screen may be used in an absolute sense, so that compounds that pass a certain criterion are termed positives, whereas those that fail to meet the criterion are termed negatives. Compounds that pass, but should have failed, are false positives. In general, false positives are tolerable, if they are not too numerous, because they will be rectified later. Compounds that fail, but should have passed, are false negatives.

False negatives are lost forever, if the failure eliminates them from further testing.

All screening procedures are based on assumptions of analogy. They have different degrees on relevance or predictability. Studies in phase II of clinical trials will predict with high probability the results in large clinical trails. But even there is the possibility of false positive or false negative results. The relevance of a test is much lower in the early pharmacological tests, such as used in high throughput screening. Generally, the relevance is inversely proportional to the simplicity of the test.

In any case, one is confronted with the problem of false positive results (first type error) and false negative results (second type error).

In each step two sources of error for **false positive results** have to be taken in account:

1. a = error of the first type due to the model
2. α = error of the first type due to statistics.

For definition: In the error of the first type a compound is considered to be active, but is actually ineffective. This type of error will be clarified during further development, after negative clinical trials at the latest.

However, there are also two sources of error for **false negative results**:

1. b = error of the second type due to the model
2. β = error of the second type due to statistics.

In the error of the second type a compound is considered to be ineffective, but is actually effective.

This type of error will never be clarified; an effective drug has just been missed. Perhaps another investigator will test this compound under different aspects.

The statistical errors derive from the fact that a pharmacological test is performed only several times or in a limited number of animals. One can specify the probability that a decision made is incorrect, i.e. a drug candidate is erroneously identified as effective, although it is actually ineffective. Usually this risk is set to 5% ($P < 0.05$) and is called the statistical error of the first type or type-I error. The error of the second type or type-II error is connected to the type-I error by statistical rules.

Usually, screening is performed sequentially. Tests in high throughput screening are followed by tests in isolated organs, then in small animals, and special tests in higher animals, until the compound is recommended for further development and for studies in human beings. From each step errors of type I, but also from type II, arise. As a consequence, many effective compounds will be lost.

There are two ways to circumvent this obstacle: Either to increase the number of compounds entering the screening procedure dramatically, and hoping for a reasonable number of true positives and accepting a high rate of false negative results (White 2000) as followed in the ultra high throughput screening, or to perform tests with high relevance, meaning tests with high predictive value in whole animals at an early stage (Vogel and Vanderbeeke 1990).

Zhang et al. (2000) studied the role of false negative results in high throughput screening procedures. They presented a statistical model system that predicts the reliability of hits from a primary test as affected by the error in the assay and the choice of the hit threshold. The hit confirmation rate, as well as false positive (representing substances that initially falls above the hit limit but whose true activity are below the hit limit) and false negative (representing substances that initially fall below the hit limit but whose true activity are in fact greater than the hit limit) rates have been analyzed by computational simulation.

The problem of type-II errors, i.e. false negative results, also exists in many other physiological and pharmacological studies (Martorana et al. 1982; Barros et al. 1991; Sandkühler et al. 1991; Waldeck 1996; Williams et al. 1997). Pollard and Howard (1986) re-investigated the staircase test, a well-accepted primary screening method for anxiolytics, and found several false negative results for clinically active anxiolytics.

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Chapter A
Cardiovascular activity[1]

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A.1 Cardiovascular analysis

A.1.1 *In vitro* methods

A.1.1.1 α_1-adrenoreceptor binding

PURPOSE AND RATIONALE

α_1-adrenoceptors are widely distributed and are activated either by norepinephrine released from sympathetic nerve terminals or by epinephrine released from the adrenal medulla. Receptor activation mediates a variety of functions, including contraction of smooth muscle, cardiac stimulation, cellular proliferation and activation of hepatic gluconeogenesis and glycolysis. In the CNS, the activation of α_1-adrenoceptors results in depolarization and increased neuronal firing rate.

The α-adrenoreceptor population of plasma membranes from rat heart ventricles consists only of the α_1-adrenoreceptor subtype. A constant concentration of the radioligand ^{3}H-prazosin (0.2–0.3 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of plasma membranes from rat heart ventricles. If the test drug exhibits any affinity to α-adrenoceptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent is the test drug. The assay is used to evaluate the binding characteristics of drugs at the α_1-adrenoceptor.

PROCEDURE

Solutions

preparation buffer A:		
Tris-HCl	5	mM
$MgCl_2 \times 6\,H_2O$	1	mM
D(+)-sucrose	250	mM
pH 7.4		
preparation buffer B (= rinse buffer):		
Tris-HCl	50	mM
$MgCl_2 \times 6\,H_2O$	10	mM
pH 7.4		
incubation buffer:		
Tris-HCl	50	mM
$MgCl_2 \times 6\,H_2O$	10	mM
ascorbic acid	1.6	mM
catechol	0.3	mM
pH 7.4		

radioligand:
^{3}H-prazosin × HCl
specific activity
0.37–1.11 TBq/mmol
(10–30 Ci/mmol) (NEN)

Tissue preparation
Male Sprague-Dawley rats (200–300 g) are sacrificed by decapitation and the dissected hearts are placed in ice-cold preparation buffer A. After removal of the atria, ventricles (approx. 30 g from 40 rats) are minced with a scalpel into 2–3 mm pieces.

Membrane preparation
Ventricles are homogenized by Ultra-Turrax (1 g tissue/20 ml preparation buffer A), the homogenate is filtered through gauze, and centrifuged at 2 000 *g* (4 °C) for 10 min. The pellets are discarded, the supernatant is collected, and centrifuged again at 40 000 *g* for 20 min. The resulting pellets are resuspended in approx. 300 ml preparation buffer B, homogenized by Ultra-Turrax and centrifuged as before. The final pellets are dissolved (by Ultra-Turrax) in preparation buffer B, corresponding to 1 g ventricle wet weight/4 ml buffer. The membrane suspension is immediately stored in aliquots of 5–20 ml at –77 °C. Protein content of the membrane suspension is determined according to the method of Lowry et al. with bovine serum albumin as a standard.

At the day of the experiment the required volume of the membrane suspension is slowly thawed and centrifuged at 40 000 *g* (4 °C) for 20 min. The pellets are resuspended in a volume of ice-cold rinse buffer, yielding a membrane suspension with a protein content of 1.0–1.5 mg/ml. After homogenization by Ultra-Turrax, the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental course
For each concentration samples are prepared in triplicate.

The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation experiments
total binding:
- 50 µl ^{3}H-prazosin
  (12 concentrations, 5×10^{-11}–5×10^{-9} M)
- 50 µl incubation buffer

non-specific-binding:
- 50 µl ^{3}H-prazosin
  (4 concentrations, 5×10^{-11}–5×10^{-9} M)
- 50 µl phentolamine (10^{-5} M)

Competition experiments
- 50 µl ^{3}H-prazosin
  (1 constant concentration, $2–3 \times 10^{-10}$ M)
- 50 µl incubation buffer without or with non-labeled test drug (15 concentrations, 10^{-10}–10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (1.0–1.5 mg protein/ml). The samples are incubated for 30 min in a shaking bath at 25 °C. The reaction is stopped by withdrawing the total incubation volume by rapid vacuum filtration over glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free activity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

EVALUATION
The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding – non-specific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-prazosin versus non-labeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D\,{}^3\mathrm{H} \times IC_{50}}{K_D\,{}^3\mathrm{H} + [{}^3\mathrm{H}]}$$

IC_{50} = concentration of the test drug, which competes 50% of specifically bound ^{3}H-prazosin in the competition experiment
$[^3\mathrm{H}]$ = concentration of ^{3}H-prazosin in the competition experiment.
$K_D\,{}^3\mathrm{H}$ = dissociation constant of ^{3}H-prazosin, determined from the saturation experiment.

The K_i-value of the test drug is the concentration, at which 50% of the receptors are occupied by the test drug. The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

Standard data: phentolamine $K_i = 2–4 \times 10^{-8}$ mol/l

MODIFICATION OF THE METHOD
Binding of ^{3}H-WB 4101 to α_1-adrenergic receptors in brain is used to test hypotensive activity as possible side effect of neuroleptic drugs. The test E.5.1.6 is described in the chapter on neuroleptic activity.

SUBTYPES OF THE α_1-ADRENOCEPTOR

Several subtypes of the α_1-adrenoceptor have been identified by pharmacological means (α_{1A} and α_{1B}, α_{1C}, α_{1D}; α_{1H}, α_{1L} and α_{1N} adrenoceptors; Endoh et al. 1992; García-Sáinz et al. 1992, 1993; Ohmura et al. 1992; Regan and Cotecchia 1992; Satoh et al. 1992; Schwinn and Lomasney 1992; Veenstra et al. 1992; Aboud et al. 1993; Oshita et al. 1993; Vargas et al. 1993; Ruffolo et al. 1994; Minneman and Esbenshade 1994; Alexander et al. 2001) or by recombinant technology ($\alpha_{1a/d}$, α_{1b}, α_{1c} adrenoceptors). They correspond to the pharmacologically defined α_{1A}, α_{1B},and α_{1D} adrenoceptors in native tissues (Bylund et al. 1994, 1998; Hieble et al. 1995; Graham et al. 1996; Hieble and Ruffolo 1996; Alexander et al. 2001).

Binding of the radioligand [^{3}H]-prazosin to the α_{1A}-adrenoceptor subtype can be measured in membranes prepared from male Wistar rat submaxillary glands (Michel et al. 1989).

Binding of the radioligand [^{3}H]-prazosin to the α_{1B}-adrenoceptor subtype can be measured in membranes prepared from male Wistar rat livers (Adolfo et al. 1989).

Decreased blood pressure response in mice deficient of the α_{1b}-adrenergic receptor was found by Cavalli et al. (1997).

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A.1.1.2 α_2-adrenoreceptor binding

PURPOSE AND RATIONALE

α_2-adrenoceptors are widely distributed and are activated by norepinephrine released from sympathetic nerve terminals or by epinephrine released from the adrenal medulla or from some neurons in the CNS. The most extensively characterized action is the prejunctionally mediated inhibition of the release of neurotransmitters from many peripheral and central neurons. α_2-adrenoceptors are also present at postjunctional sites, where they mediate actions such as smooth muscle contraction, platelet aggregation and inhibition of insulin secretion. Activation of postsynaptic α_2-adrenoceptors in the brainstem results in an inhibition of sympathetic outflow in the periphery.

Clonidine is a centrally-acting antihypertensive agent, which lowers blood pressure mostly through reducing sympathetic tone by acting at the nucleus tractus solitarius in the brain stem (Kobinger and Walland 1967). Clonidine can, however, act at both peripheral and central α_2-receptors. Peripherally administered clonidine causes a brief increase in blood pressure followed by a prolonged decrease (Rand and Wilson 1968). Functional studies (^{3}H-NE release) indicate a presynaptic mechanism for clonidine (Langer 1977, 1981; Starke 1977). However, lesioning studies fail to confirm a presynaptic location for clonidine receptors in either the CNS or periphery (U'Prichard et al. 1979; Bylund and Martinez 1981; U'Prichard et al. 1980). No change in clonidine receptor sites was seen after 6-hydroxydopamine lesions in cerebral cortex. This may be due to the fact that α_2-receptors are both pre- and postsynaptic (Hieble et al. 1988).

Alpha-adrenergic agonists most potently displace ^{3}H-clonidine. Ergot compounds, dopamine agonists and mianserin are also fairly potent (U'Prichard et al. 1977). A survey on functions mediated by alpha-2 adrenergic receptors was given by Ruffolo et al. (1988) and on the role of neurotransmitters in the central regulation of the cardiovascular system by McCall (1990). Although clonidine relieves the autonomic symptoms of morphine withdrawal (Gold et al. 1978), there is no evidence for a direct α_2/opiate-receptor interaction.

The purpose of this assay is to assess the interaction of hypotensive agents with central α_2-receptors and determine possible clonidine-like mechanisms of action. Clonidine binding may also be relevant to the activity of other classes of drugs such as antidepressants that interact with α_2-receptors.

PROCEDURE

Reagents

1. Tris buffer pH 7.7
   a) 57.2 g Tris HCl q.s. to 1 liter
      (0.5 M Tris buffer, pH 7.7)
      16.2 g Tris base
   b) make a 1:10 dilution in distilled H_2O
      (0.05 M Tris buffer, pH 7.7)
2. Tris buffer containing physiological ions
   a) Stock buffer

NaCl	7.014 g
KCl	0.372 g
$CaCl_2$	0.222 g
$MgCl_2$	0.204 g

   q.s to 100 ml in 0.5 M Tris buffer
   b) Dilute 1:10 in distilled H_2O.
      This yields 0.05 M Tris HCl, pH 7.7; containing NaCl (120 mM), KCl (5 mM), $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM)
3. [4-^{3}H]-Clonidine hydrochloride (20–30 Ci/mmol) is obtained from New England Nuclear.
   For IC_{50} determinations: ^{3}H-Clonidine is made up to a concentration of 120 nM and 50 µl are added to each tube (yielding a final concentration of 3 nM in the 2 ml volume assay).
4. Clonidine-HCl is obtained from Boehringer Ingelheim.
   A stock solution of 0.1 mM clonidine is made up to determine non-specific binding. This yields a final concentration of 1 µM in the assay (20 µl to 2 ml).
5. Test compounds:
   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, so that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay and higher or lower concentrations can be used, depending on the potency of the drug.

Tissue preparation

Male Wistar rats are sacrificed by decapitation and the cortical tissue is rapidly dissected. The tissue is homogenized in 50 volumes of 0.05 M Tris buffer pH 7.7 (buffer 1b) with the Brinkman Polytron, and centrifuged at 40 000 *g* for 15 min. The supernatant is discarded and the final pellet rehomogenized in 50 vol-

umes of buffer 2b. This tissue suspension is then stored on ice. The final tissue concentration is 10 mg/ml. Specific binding is 1% of the total added ligand and 80% of total bound ligand.

Assay

100 µl	0.5 M Tris – physiological salts pH 7.7 (buffer 2a)
830 µl	H_2O
20 µl	Vehicle (for total binding) or 0.1 mM clonidine (for nonspecific binding) or appropriate drug concentration.
50 µl	^{3}H-clonidine stock solution
1 000 µl	tissue suspension.

Tissue homogenates are incubated for 20 min at 25 °C with 3 nM ^{3}H-clonidine and varying drug concentrations, and immediately filtered under reduced pressure on Whatman GF-B filters. The filters are washed with 3 five ml volumes of 0.05 M Tris buffer pH 7.7, and transferred to scintillation vials. Specific clonidine binding is defined as the difference between total bound radioactivity and that bound in the presence of 1 µM clonidine.

EVALUATION

IC_{50} calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

MODIFICATIONS OF THE METHOD

Perry and U'Prichard (1981) described [^{3}H]rauwolscine (α-yohimbine) as a specific radioligand for brain α_2-adrenergic receptors.

Goldberg and Robertson (1983) reviewed yohimbine as a pharmacological probe for the study of the α_2-adrenoreceptor.

Pimoule et al. (1983) characterized [^{3}H]RX 781094 [(imidazolinyl-2)-2 benzodioxane-1,4] as a specific α_2-adrenoceptor antagonist radioligand.

Murphy and Bylund (1988) characterized alpha-2 adrenergic receptors in the OK cell, an opossum kidney cell line.

Binding of the radioligand [^{3}H]-rauwolscine to the α_{2A}-adrenoceptor subtype can be measured in membranes prepared from rabbit spleens (Michel et al. 1989).

Binding of the radioligand [^{3}H]-yohimbine to the α_{2B}-adrenoceptor subtype can be measured in membranes prepared from male Wistar rat kidney cortices (Connaughton and Docherty 1989).

SUBTYPES OF THE α_2-ADRENOCEPTOR

Using ^{3}H-rauwolscine as ligand Broadhurst et al. (1988) studied the existence of two alpha$_2$-adrenoreceptor subtypes.

Bylund et al. (1988) used [^{3}H]-yohimbine and [^{3}H]-rauwolscine to study alpha-2A and alpha-2B adrenergic subtypes in tissues and cell lines containing only one subtype.

Brown et al. (1990) found that [^{3}H]-yohimbine labels at α_{2A}- and α_{2B}-adrenoceptors whereas [^{3}H]-idazoxan labels the α_{2A}-adrenoceptor and, in addition, an imidazoline binding site.

Several subtypes of the α_2-adrenoceptor have been identified by pharmacological means (α_{2A}-, α_{2B}-, α_{2C}-, and α_{2D}-adrenoceptors; Ruffulo 1990; Uhlén and Wikberg 1990; Gleason and Hieble 1992; Satoh and Takayanagi 1992; Takano et al. 1992; Ruffolo et al. 1993) or by recombinant technology as α_{2a}-, α_{2b}-, α_{2c}-adrenoceptors (Bylund et al. 1994; Hieble et al. 1995; Hieble and Ruffolo 1996; Alexander et al. 2001).

Gleason and Hieble (1992) reported that the α_2-adrenoreceptors of the human retinoblastoma cell line (Y79) may represent an additional example of the α_{2C}-adrenoceptor.

Marjamäki et al. (1993) recommended the use of recombinant human α_2-adrenoceptors to characterize subtype selectivity of antagonist binding.

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A.1.1.3 Electrically stimulated release of [^{3}H]norepinephrine from brain slices

PURPOSE AND RATIONALE

The existence of presynaptic receptors which regulate the evoked release of neurotransmitters has been functionally demonstrated in both peripheral and central nervous system (Langer 1981; Starke 1981; Raiteri et al. 1984). Presynaptic adrenergic α_2-receptors regulate the evoked release of norepinephrine, comprising a short negative feedback loop. Alpha-2 agonists, such as clonidine and guanabenz, inhibit evoked release and alpha-2 antagonists, such as yohimbine and idazoxan, enhance evoked release.

The assay is used as a biochemical screen for agents which enhance or inhibit release of [^{3}H]norepinephrine (^{3}H-NE) and is particularly useful for testing receptor function of α_2-adrenergic agonists and antagonists.

The procedures used emphasize delicate care of slices. By treating slices with great care, one is able to incubate at low tracer concentrations of ^{3}H-NE (25 nM), thus minimizing nonspecific labeling of releasable pools other than those in noradrenergic nerve terminals. It also permits the use of low (and more physiological) stimulation parameters, which allow the neurons to recover easily between stimulations and do not flood the synaptic cleft with released NE, which would compete with any applied drug thus decreasing sensitivity.

PROCEDURE

This assay is based on the method described by Zahniser et al. (1986).

A Reagents

1. Krebs-Henseleit bicarbonate buffer, pH 7.4 (KHBB):

NaCl	118.4 mM
KCl	4.7 mM
$MgSO_4 \times 7\ H_2O$	1.2 mM
KH_2PO_4	2.2 mM
$NaHCO_3$	24.9 mM
$CaCl_2$	1.3 mM
dextrose (added prior to use)	11.1 mM

   The buffer is aerated for 60 min with 95% O_2, 5% CO_2 on ice and pH is checked.

2. Levo-[Ring-2,5,6-^{3}H]-norepinephrine (specific activity 40–50 Ci/mmol) is obtained from New England Nuclear.

   The final desired concentration of ^{3}H-NE is 25 nM. 0.125 nmol is added to 5 ml KHBB.

3. Test compounds

   For most assays, a 1 mM stock solution of the test compound is made up in a suitable solvent and diluted such that the final concentration in the assay is 1 µM. Higher or lower concentrations may be used depending on the potency of the drug.

B Instrumentation

Neurotransmitter release apparatus consisting of:

- oscilloscope B8K, Precision Model 1420, dual-trace microscope (Dynascan Corp.)
- constant current unit, Grass model CCU1 (Grass Instr. Co.)
- stimulator, model S44, solid state square wave stimulator (Grass Instr. Co.)
- pump, Watson-Marlow, model 502 SHR, standard drive module; model 501 M multichannel pumphead (Bacon Technical Instr.)
- circulator, Haake D8 immersion circulator (Haake Buchler Instr. Inc.)
- fraction collector, Isco Retriever IV fraction collector (Isco Inc.)

C Tissue Preparation

Male Wistar rats (100–150 g) are decapitated, cortical tissue removed on ice and 0.4 mm slices are prepared with a McIlwain tissue chopper. The slices are made individually and removed from the razor blade by twirling an artist's paint brush underneath the slice. Care should be taken not to compress the slice or impale it on the bristles. The slices are placed in cold, oxygenated buffer (10–20 ml) and incubated at 35 °C for 30 min under oxygen. After this incubation, the buffer is decanted, leaving the slices behind. Then 5 ml of cold oxygenated buffer is added, and enough [^{3}H]NE to bring the final concentration to 25 nM. This is then incubated and shaken for 30 min at 35 °C under oxygen. After this step, the buffer is decanted and the "loaded" slices are rapidly placed on the nylon mesh in the stimulation chambers using a cut-off pipetman tip.

D Assay

To establish a stable baseline, control buffer is pumped through the chamber for 1 h at a flow rate of 0.7 ml/min before the first stimulation. One hour is allowed to pass before the second stimulation. When drugs are used, each concentration is prepared in a separate flask in control buffer and allowed to equilibrate with the tissue slice 20 min before the second stimulation. The experiment is stopped 40 min after the second stimulation.

Stimulation parameters are set at 5 Hz (2 ms duration) for 60 s, with 1 ms delay and voltage setting of 440 SIU (250 Ω).

After the experiment is completed, the chambers are washed with distilled water for at least 20 min, then 200 ml of 20% methanol in distilled water, then distilled water again for at least 20 min.

EVALUATION

After conversion of dpm, percent fractional release is calculated for each fraction, using the spreadsheet program.

Percent fractional release is defined as the amount of radiolabeled compound released divided by the amount present in the tissue at that moment in time. "Spontaneous release" (SP) values are the average of the two fractions preceding and the first fraction in that range after the stimulation period. "Stimulated" (S) are the summed differences between the percent fractional release during stimulation and the appropriate SP value.

The effects of drugs can be reported as S_2/S_1 ratios. To normalize the data, drug effects can be estimated by first calculating S_2/S_1 values for control and drug-treated slices and then expressing the S_2/S_1 value for the drug-treated slices as a percentage of the S_2/S_1 value for the control slices for each experiment. Each condition should be tested in slices from each animal.

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A.1.1.4 Imidazoline receptor binding

PURPOSE AND RATIONALE

Imidazoline receptors constitute a family of nonadrenergic high-affinity binding sites for clonidine, idazoxan, and allied drugs. Drugs selectively binding to imidazoline receptors are expected to have less side effects than clonidine (Ernsberger et al. 1992, 1997; Molderings et al. 1992; Limon et al. 1992). One major subclass, the I_1 receptors, being mainly distributed in the brain and brain stem, partly mediates the central hypotensive action of clonidine-like drugs. The I_2 receptors, an other subclass, are mitochondrial, not G protein coupled, and have diversified functions. They may be involved in neuroprotection for cerebral ischemia. Two binding sites of [^{3}H]p-aminoclonidine, α_2-adrenoceptors and imidazoline binding sites, could be separated (Ernsberger et al. 1987; Bricca et al. 1988; Kamisaki et al. 1990). At least 3 subtypes of imidazoline/guanidinium-receptive sites have been found by photoaffinity labeling (Lanier et al. 1993).

Several endogenous ligands for imidazoline receptors, collectively termed clonidine displacing substances (CDSs), have been detected in tissues and serum (Reis et al. 1995; Chan et al. 1997).

An endogenous substance with clonidine-like properties originally isolated from brain which binds selectively to imidazoline receptors was described by Atlas and Burstein (1984), Ernsberger et al. (1988), Atlas (1991), Meeley et al. (1992), Dontenwill et al. (1992, Ragunathan and Reis 1996). The endogenous substance agmatine, a decarboxylated arginine, may be the physiological agonist at imidazoline receptors acting as neurotransmitter (Li et al. 1994; Gonzales et al. 1996; Head et al. 1997; Herman 1997; Reis and Regunathan 2000). Further candidates of endogenous ligands are discussed (Reis and Ragunathan 1998).

A critical review on imidazoline binding sites is given by Eglen et al. (1998).

PROCEDURE

Tissue preparation

Whole bovine brains and adrenal glands are obtained from a local slaughterhouse. The lateral medulla oblongata is isolated by a sagittal section through the lateral margin of the pyramids and then bisected. The ventral half is defined as the ventrolateral medulla.

Fresh bovine adrenal glands are perfused retrogradely through the adrenal vein twice with 25 ml ice-cold Krebs-Henseleit bicarbonate buffer. The glands are perfused again with 25 ml ice-cold Krebs-Henseleit buffer containing 0.025% collagenase (type I, Sigma Chemical), incubated at room temperature for 1 h, then perfused with 25 ml fresh buffer containing collagenase and incubated for 30 min at 35 °C. The digested glands are split, and the medulla is removed from the cortex. Adrenal medullae are minced and incubated while being stirred for 30 min at 37 °C. The digest is filtered and centrifuged at 200 *g* for 30 min at 20 °C. The cell pellet is resuspended in 30 ml Krebs' solution without collagenase, recentrifuged, flash-frozen, and stored at –70 °C.

Membrane preparation

Fresh bovine ventrolateral medulla and collagenase-digested rat renal medulla are homogenized with a Polytron (Tekmar Tissumizer; setting 80 for 15 s twice) in 20 vol of ice-chilled HEPES-buffered isotonic sucrose (pH 7.4) containing the protease inhibitors 1,10-phenanthroline (100 µM) and phenylmethyl-sulfonyl fluoride (50 µM). Bovine adrenomedullary chromaffin cells are homogenized in 15 ml HEPES-buffered isotonic sucrose by 10 strokes in a glass/glass handhold homogenizer. The homogenates are centrifuged at 1 000 *g* for 5 min at 4 °C to remove nuclei and debris. The pellets (P1) are resuspended in 20 ml of homogenization buffer and centrifuged again at 1 000 *g* for 5 min. The supernatants are centrifuged at 48 000 *g* for 18 min at 4 °C, and the resulting pellet (P2) is re-suspended in 10–25 vol 50 mM Tris-HCl buffer (pH 7.7) containing 5 mM EDTA. After recentrifugation at 48 000 *g* for 18 min, the resulting membrane pellet is resuspended in Tris-HCl containing 25 mM NaCl, preincubated for 30 min at 25 °C, chilled on ice, centrifuged again, resuspended a final time in Tris-HCl alone, centrifuged, flash-frozen, and stored at –70 °C.

Binding assays

For determination of specific binding to I_1-imidazoline sites and α_2-adrenergic receptors radioligand binding assays are performed with [^{3}H]clonidine, [^{3}H]*p*-iodoclonidine, or [^{3}H]moxonidine. Membranes are slowly thawed and resuspended in Tris-HCl or Tris-HEPES buffer (pH 7.7, 25 °C). Assays are conducted in a total volume of 250 µl in polypropylene 96 well plates (Beckman Macrowell). Each well contains 125 µl membrane suspension, 25 µl radioligand, and 100 µl drug or vehicle. Incubations are initiated by the addition of membrane suspension and carried out for 40 min at 25 °C. Nonspecific binding is defined in the presence of either piperoxan or phentolamine (0.1 mM), which are imidazoline-adrenergic agents. Specific α_2-adrenergic binding is defined by epinephrine (0.1 mM). In experiments with catecholamines, all samples contain ascorbic acid in a final concentration of 0.001%. Incubations are terminated by vacuum filtration over Reeves-Angel or Whatman GF/C fiberglass filters us-

ing a cell harvester (Brandel). The filters are washed four times with 5 ml ice-cold Tris-HCl, placed in scintillation vials, covered with 4 ml scintillation cocktail and counted at 50% efficiency. Protein is assayed by a modified Lowry et al. method (Peterson 1977) using a deoxycholate-trichloroacetic acid protein precipitation technique which provides a rapid quantitative recovery of soluble and membrane proteins from interfering substances even in very dilute solutions. Sodium dodecyl sulfate is added to alleviate possible nonionic and cationic detergent and lipid interferences, and to provide mild conditions for rapid denaturation of membrane and proteolipid proteins.

EVALUATION

Data are obtained as disintegrations per min and transferred to the Equilibrium Binding Data Analysis program (McPherson 1985). Then, several experiments are analyzed simultaneously with the LIGAND program for non-linear curve fitting (Munson and Rodbard 1980). IC_{50} values are estimated from inhibition curves by non-linear curve fitting (Mutolsky and Ransnas 1987). Protein assay data are also analyzed by non-linear curve-fitting (McPherson 1985).

MODIFICATIONS OF THE METHOD

Tesson et al. (1991) defined the subcellular localization of imidazoline-guanidinium-receptive sites by performing binding studies with the radioligand [^{3}H]idazoxan.

Lanier et al. (1993) visualized multiple imidazoline/guanidinium-receptive sites with the photoaffinity adduct 2-[3-azido-4-[^{125}I]iodo-phenoxy]methyl imidazoline.

Molderings et al. (1991) characterized imidazoline receptors involved in the modulation of noradrenaline release in the rabbit pulmonary artery pre-incubated with [^{3}H]noradrenaline.

Molderings and Göthert (1995) determined electrically or K^+-evoked tritium overflow from superfused rabbit aortic strips pre-incubated with [^{3}H]noradrenaline in order to characterize presynaptic imidazoline receptors which mediate noradrenaline release and compared them with I_1- and I_2-imidazoline radioligand binding sites.

Ernsberger et al. (1995) described optimization of radioligand binding assays for I_1 imidazoline sites.

Munk et al. (1996) reported the synthesis and pharmacological evaluation of a potent imidazoline-1 receptor specific agent.

Piletz et al. (1996) compared the affinities of several ligands for [^{125}I]p-iodoclonidine binding at human platelet I_1 imidazole binding sites.

Several selective ligands for imidazoline I_2 receptors have been identified, such as:

- LSL 60101 (Alemany et al. 1995; Menargues et al. 1995),
- RS-45041-190 (MacKinnon et al. 1995; Brown et al. 1995),
- RX801077 (= 2-BFI = 2-(2-benzofuranoyl)-2-imidazoline and analogues (Jordan et al. 1996; Lione et al. 1996; Alemany et al. 1997; Hosseini et al. 1997; Wiest and Steinberg 1997; Hudson et al. 1997).

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A.1.1.5 β-adrenoreceptor binding

PURPOSE AND RATIONALE

β-adrenoceptors are widely distributed, found at both central and peripheral sites, and are activated either via norepinephrine released from sympathetic nerve terminals or via epinephrine released from the adrenal medulla. Important physiological consequences of β-adrenoceptor activation include stimulation of cardiac rate and force, relaxation of vascular, urogenital and bronchial smooth muscle, stimulation of renin secretion from the juxta-glomerular apparatus, stimulation of insulin and glucagon secretion from the endocrine pancreas, stimulation of glycogenolysis in liver and skeletal muscle and stimulation of lipolysis in the adipocyte.

Three β-adrenoceptor proteins have been cloned, and the characteristics of these recombinant receptors correspond with those of the three well characterized β-adrenoceptors on native tissue, designated as β_1, β_2 and β_3. The possible roles of β_3-adrenoceptors in the cardiovascular system were discussed by Gauthier et al. (2000). An additional β-adrenoceptor modulatingcardiac contractility has been designated as the β_4-adrenoceptor (Kaumann et al. 1998).

The β-adrenoceptor population of plasma membranes from bovine heart ventricles consists of 75–80% β_1- and 20–25% β_2-adrenoceptors. The use of this tissue allows a parallel investigation of the binding characteristics of drugs at both the β_1- and β_2-adrenoceptors. Both, the β_1- and β_2-adrenoceptors coexist in rat ventricular myocytes, but stimulation of these receptor subtypes elicits qualitatively different cell responses at the levels of ionic channels, the myofilaments, and sarcoplasmic reticulum (Xiao and Lakatta 1993).

A constant concentration of the radioligand ^{3}H-dihydroalprenolol (^{3}H-DHA) (4–6 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of plasma membranes from bovine heart ventricles. If the test drug exhibits any affinity to ß-adrenoceptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more effective is the test drug.

PROCEDURE

Materials and solutions

preparation buffer:

Tris-HCl	5	mM
$MgCl_2 \times 6\ H_2O$	1	mM
D(+)-sucrose	250	mM
pH 7.4		

310 mOsm sodium phosphate buffer:
pH 7.4

rinse buffer:

Tris-HCl	50	mM
$MgCl_2 \times 6\ H_2O$	10	mM
pH 7.4		

incubation buffer:

Tris-HCl	50	mM
$MgCl_2 \times 6\ H_2O$	10	mM
ascorbic acid	1.6	mM
catechol	0.3	mM
pH 7.4		

radioligand:
(–)^{3}H-dihydroalprenolol × HCl
(^{3}H-DHA) specific activity 1.48–2.59 TBq/mmol (40–70 Ci/mmol) (NEN)
for inhibition of ^{3}H-dihydroalprenolol binding in non-specific binding experiments:
(–)isoprenaline(+)-bitartrate salt (Sigma)

Bovine hearts are obtained freshly from the local slaughter house. The lower part of the left ventricle from 5 hearts is separated and kept in ice-cold preparation buffer. In the laboratory, approx. 60 g wet weight from the five ventricle pieces are minced with a scalpel into 2–3 mm pieces.

Membrane preparation

Ventricles are homogenized by Ultra-Turrax (1 g tissue/10 ml buffer), the homogenate is filtered through gauze and centrifuged at 500 *g* (4 °C) for 10 min. The pellets are discarded, the supernatant is collected, and centrifuged at 40 000 *g* for 20 min. The resulting pellets are resuspended in approx. 300 ml 310 mOsm sodium phosphate buffer, homogenized by Ultra-Turrax, and centrifuged as before. The final pellets are dissolved (by Ultra-Turrax) in sodium phosphate buffer corresponding to 1 g ventricle wet weight/2 ml buffer.

The membrane suspension is immediately stored in aliquots of 5–20 ml at –77 °C. Protein concentration of the membrane suspension is determined according to the method of Lowry et al. with bovine serum albumin as a standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed and centrifuged at 40 000 *g* (4 °C) for 20 min. The pellets are resuspended in a volume of ice-cold rinse buffer, yielding a membrane suspension with a protein content of approx. 2.0 mg/ml. After homogenizing by Ultra-Turrax, the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental course

All incubation samples are performed in triplicate.

The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation experiments

total binding:

- 50 µl ^{3}H-DHA
  (12 concentrations, 3×10^{-10}–4×10^{-8} M)
- 50 µl incubation buffer

non-specific-binding:

- 50 µl ^{3}H-DHA
  (4 concentrations, 3×10^{-10}–4×10^{-8} M)
- 50 µl (–)isoprenaline (10^{-5} M)

Competition experiments

- 50 µl ^{3}H-DHA
  (1 constant concentration, $4–6 \times 10^{-9}$ M)
- 50 µl incubation buffer without or with non-labeled test drug
  (15 concentrations 10^{-10}–10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (approx. 2 mg protein/ml). The samples are incubated for 60 min in a shaking water bath at 25 °C. The reaction is stopped by rapid vacuum filtration of the total incubation volume over glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free activity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a Packard liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding – non-specific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-DHA versus non-labeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D\ ^3\mathrm{H} \times IC_{50}}{K_D\ ^3\mathrm{H} + [^3\mathrm{H}]}$$

IC_{50} = concentration of the test drug, which competes with 50% of specifically bound ^{3}H-DHA in the competition experiment

$[^3\mathrm{H}]$ = concentration of ^{3}H-DHA in the competition experiment.

$K_D\ ^3\mathrm{H}$ = dissociation constant of ^{3}H-DHA, determined from the saturation experiment.

The K_i-value of the test drug is the concentration, at which 50% of the receptors are occupied by the test drug.

The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

Standard data

propranolol hydrochloride $K_i = 6–8 \times 10^{-9}$ mol/l

MODIFICATIONS OF THE METHOD

Abrahamsson et al. (1988) performed a receptor binding study on the β_1- and β_2-adrenoceptor affinity of atenolol and metoprolol in tissues from the rat, the guinea pig and man with various radioligands, such as $[^{125}\mathrm{I}](\pm)$hydroxybenzylpindolol, $[^{125}\mathrm{I}](-)$pindolol, $[^3\mathrm{H}](-)$-dihydroalprenolol, and $[^3\mathrm{H}](-)$CGP 12177.

Fleisher and Pinnas (1985) used specific binding of $(-)[^3\mathrm{H}]$dihydroalprenolol to rat lung membranes for *in vitro* studies on the relative potency of bronchodilator agents.

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A.1.1.6 β_1-adrenoreceptor binding

PURPOSE AND RATIONALE

β-adrenergic receptors were differentiated from α-receptors (Ahlquist 1948) and subsequently divided into 2 distinct subtypes, β_1 and β_2 (Lands et al. 1967) based on differing pharmacology in different tissues. β-receptors have been labelled in a number of tissues including heart, lung, erythrocytes and brain using the β-agonists [^{3}H]-epinephrine (U'Prichard et al. 1978), or [^{3}H]-hydroxybenzylisoproterenol (Lefkowitz and Williams 1977) or the β-receptor antagonists [^{3}H]-alprenolol (Mukherjee et al. 1975), [^{3}H]-dihydroalprenolol (DHA) (U'Prichard et al. 1978; Bylund and Snyder 1976) and (^{125}I)-iodohydroxypindolol (Weiland et al. 1980). DHA is a potent β-antagonist (Mukherjee et al. 1975), which labels both β_1 and β_2 adrenergic receptors. The binding characteristics of this ligand in brain were described by Bylund and Snyder (1976), who showed that antagonists competed potently and agonists less potently although stereospecificity was maintained. The pharmacology of binding was consistent with β_1-receptor occupancy. Lesioning studies (Wolfe et al. 1982), combined with non-linear regression analysis of data have shown that while β-receptors in rat cerebellum are primarily of the β_2 sub-type, the β_1 occurring in rat cerebral cortex are physiologically more significant. The assay can be used to evaluate the direct interaction of drugs with β-receptors labelled by [^{3}H]-dihydroalprenolol.

PROCEDURE

Reagents

Tris buffer, pH 8.0
- a) 44.4 g Tris HCl q.s. to 1 liter (0.5 M Tris, pH 8.0) 26.5 g Tris base
- b) Dilute 1:10 in distilled water. (0.05 M Tris, pH 8.0)

2. (–)-[propyl-1,2,3-^{3}H] Dihydroalprenolol hydrochloride (45–52 Ci/mmol) is obtained from New England Nuclear.

   For IC_{50} determinations: A stock solution of 20 nM ^{3}H-DHA is made up in distilled H_2O and 50 µl is added to each tube (this yields a final concentration of 1 nM in the 1 ml assay).
3. (±)-propranolol HCl is obtained from Ayerst.

   A 1 mM propranolol stock solution is made up in distilled water and further diluted 1:20 in distilled water to give 50 µM propranolol solution. Twenty µl of dilute stock solution is added to 3 tubes to determine nonspecific binding (yields a final concentration of 1 µM in a 1 ml assay).
4. Test compounds:

   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the compound.

Tissue preparation

Male rats are decapitated and the brains rapidly removed. The cerebral cortices are dissected free, weighed and homogenized in 50 ml of ice-cold 0.05 Tris buffer, pH 8.0. This homogenate is centrifuged at 40 000 *g*, the supernatant decanted and the pellet resuspended and recentrifuged at 40 000 *g*. The final pellet is resuspended in the initial volume of fresh 0.05 Tris buffer, pH 8.0. This tissue suspension is then stored on ice. The final tissue concentration in the assay is 10 mg/ml. Specific binding is about 3% of the total added ligand and 80% of the total bound ligand.

Assay

380 µl	H_2O
50 µl	0.5 Tris buffer, pH 8.0
20 µl	Vehicle (for total binding) or 50 µM (±) propranolol (for nonspecific binding) or appropriate drug concentration
50 µl	^{3}H-DHA stock solution
500 µl	tissue suspension.

The tissue homogenates are incubated for 15 min at 25 °C with 1 nM ^{3}H-DHA and varying drug concentrations. With each binding assay, triplicate samples are incubated with 1 µM (±)-propranolol under identical conditions to determine nonspecific binding. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed 3 times with 5 ml of ice-cold 0.05 Tris buffer, pH 8.0. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

EVALUATION

The percent inhibition of each drug concentration is the mean of triplicate determinations. IC_{50} values are obtained by computer-derived log-probit analysis.

MODIFICATIONS OF THE METHOD

Dooley et al. (1986) recommended CGP 20712 A as a useful tool for quantitating β_1 and β_2 adrenoceptors.

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A.1.1.7 β_2-adrenoreceptor binding

PURPOSE AND RATIONALE

Lands et al. (1967) classified β-receptors into β_1 and β_2 subtypes according to differences in the action of various catecholamines. Synthesis of more selective β-antagonists has helped to confirm the existence of receptor subtypes. Based on catecholamine pharmacology and differences in the tissue distribution, it has been suggested that the β_1-receptor serves as the receptor for norepinephrine acting as a neurotransmitter and the β_2-receptor serves as a receptor for epinephrine acting as a hormone. (Nahorski 1981; Ariens and Simonis 1983; Lefkowitz et al. 1983; Minneman 1983). Since [^{3}H]-dihydroalprenolol is a nonspecific ligand, it is necessary to select a tissue which is enriched in β_2-receptors in order to convey specificity to this assay. Tissues with predominantly β_2-receptors include lung (U'Prichard et al. 1978; Ariens and Simonis 1983; Lefkowitz et al. 1983), cerebellum (Lefkowitz et al. 1983; Minneman et al. 1983), rat and frog erythrocytes (Mukherjee et al. 1975; Lefkowitz et al. 1983) and ciliary process (Nathanson 1985) whereas, forebrain, heart and avian erythrocytes are relatively enriched in the β_1-subtype (Lefkowitz et al. 1983). Due to poor binding characteristics in cerebellum, rat lung is chosen as the tissue for β_2-adrenergic receptors.

A compound with β_2-selectivity would be less likely to produce cardiac effects but more likely to produce bronchiolar constriction. The test is used to determine the affinity of compounds for the β_2-adrenergic receptor subtype. A measure of receptor subtype selectivity can be determined when data are compared with those obtained in the β_1-adrenergic assay in rat cerebral cortex.

The present nomenclature of β_1, β_2, and β_3 receptors was reviewed by Alexander et al. (2001).

PROCEDURE

Reagents

1. Tris buffers, pH 8.0
   a) 44.4 g Tris HCl q.s. to 1 liter (0.5 M Tris, pH 8.0) 26.5 g Tris base
   b) Dilute 1:10 in distilled water (0.05 M Tris, pH 8.0)
2. (–)-[propyl-1,2,3-^{3}H] Dihydroalprenolol hydrochloride (45–52 Ci/mmol) is obtained from New England Nuclear.
   For IC_{50} determinations: A stock solution of 20 nM ^{3}H-DHA is made up in distilled water and 50 µl is added to each tube (this yields a final concentration of 1 nM in the assay)
3. (±)-propranolol HCl is obtained from Ayerst.
   A 1 mM propranolol stock solution is made up in distilled water and further diluted 1:20 in distilled water to give 50 µM propranolol solution. Twenty µl of dilute stock solution are added to 3 tubes to determine nonspecific binding (yielding a final concentration of 1 µM in a 1 ml assay).
4. Test compounds:
   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the compound to be tested.

Tissue preparation

Male Wistar rats are sacrificed by decapitation and the lungs removed, weighed and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer, pH 8.0 using a Tekmar homogenizer. The homogenate is passed through a cheese cloth and centrifuged at 40 000 *g* for 15 min. The final membrane pellet is resuspended in the original volume of Tris buffer, pH 8.0, and used in the assay.

Assay

380 µl H_2O
50 µl 0.5 Tris buffer, pH 8.0
20 µl Vehicle (for total binding) or 50 µM (±)-propranolol (for nonspecific binding) or appropriate drug concentration
50 µl ^{3}H-DHA stock solution
500 µl tissue suspension.

The tissue homogenates are incubated for 15 min at 25 °C with 1 nM ^{3}H-DHA and varying drug concentrations. In each binding assay, triplicate samples are incubated with 1 µM (±)-propranolol under identical conditions to determine nonspecific binding. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed 3 times with 5 ml of ice-cold 0.05 Tris buffer, pH 8.0. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

EVALUATION

The percent inhibition of each drug concentration is the mean of triplicate determinations. IC_{50} values are obtained by computer-derived log-probit analysis.

MODIFICATIONS OF THE METHOD

Dooley et al. (1986) recommended CGP 20712 as a useful tool for quantitating β_1- and β_2-adrenoceptors.

Sarsero et al. (1998) recommended (–)[^{3}H]-CGP 12177A as radioligand for the putative β_4-adrenoceptor.

McConnell et al. (1991, 1992; Owicki and Parce 1992) used a special apparatus, the 'cytosensor microphysiometer' which measures the rate of proton excretion from cultured cells. Chinese hamster ovary cells were transfected with human β_2-adrenergic receptors. The β_2-adrenergic receptor activates adenylate cyclase resulting in an increase in the cyclic AMP concentration within the cell which can be measured as acidification. Addition of 10 µM isoproterenol, 500 µM 8-bromo cyclic AMP, or 10 µg/ml forskolin induced a reversible acidification.

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A.1.1.8 Adenosine A_1 receptor binding

GENERAL CONSIDERATIONS

Adenosine receptors belong to the class of purinoceptors (Burnstock 1972, 1981; Olsson and Pearson 1990). Purinoceptors are divided into two general types on the basis of recognized natural ligands:

P_1 receptors recognize adenosine and AMP and P_2 receptors recognize ATP and AMP. Fredholm et al. (1994), Abbracchio and Burnstock (1994, Jacobson et al. 2000) proposed a nomenclature system which is now widely accepted: two families of P2 purinoceptors, P2X ionotropic ligand-gated ion channel receptors and P2Y metabotropic G-protein-coupled receptors. The nomenclature of seven subtypes of P2X receptors and six subtypes of P2Y receptors has been agreed by the NC-IUPHAR Subcommittee (Burnstock 2001; Alexander et al. 2001).

The effects of adenosine are mediated effects through cAMP (Sattin and Rall 1970; VanCalker et al. 1978). It was discovered that adenosine could either inhibit or stimulate the formation of cAMP. The discovery of dual effects on adenylate cyclase led to the proposal of two distinct adenosine receptors referred to as the A_1 and A_2 receptors. The A_1 subtype of adenosine receptor mediates the inhibition of adenylate cyclase; whereas, the A_2 subtype mediates stimulation of adenylate cyclase. The methylxanthines are relatively nonselective inhibitors of adenosine receptor subtypes and their pharmacological properties are thought to be mostly due to antagonism of these receptors.

Comparison of adenosine receptors with other G-protein linked receptors indicates that they comprise a family of G protein coupled receptors that can be grouped by subtypes or by species. Thus, in addition to A_1 and A_2, several authors described A_{1a}, A_{1b}, A_{2a}, A_{2b}, A_3, and A_4 receptors with species dependent differences (Jacobson et al. 1992 1996; Zhou et al. 1992; Linden et al. 1993; Salvatore et al. 1993; Linden et al. 1994; Fredholm et al. 1994; Alexander et al. 2001).

PURPOSE AND RATIONALE

The purpose of this assay is to measure the affinity of test compounds for adenosine (A_1) receptors. Evidence for an A_1 adenosine receptor in the guinea pig atrium was given by Collis (1983). Adenosine plays a physiological role in many systems, including platelet aggregation, lipolysis, steroidogenesis and smooth muscle tone (Daly 1982).The vasodilatory and cardiac depressant effects of adenosine are well known. In addition to cardiovascular effects, adenosine has marked effects in the CNS including depression of electrophysiological activity (Siggins and Schubert 1981), anticonvulsant activity, analgesic properties (Ahlijanian and Takemori 1985) and inhibition of neurotransmitter release (Harms et al. 1979).

The agonist, [^{3}H]cyclohexyladenosine (CHA), has affinity for the A_1 receptor in the nanomolar concentration range and has proven to be a suitable ligand for A_1 receptor assays (Bruns et al. 1980; Bruns et al. 1986). Selective A_1 (Schingnitz et al. 1991) and A_2 antagonists (Shimada et al. 1992; Jacobson et al. 1993) have been described. Adenosine and its nucleotides have not only a cardiovascular but predominantly a cerebral activity (Phillis and Wu 1981; Daly 1982; Fredholm et al. 1982).

PROCEDURE

Reagents

1. a) 0.5 M Tris buffer, pH 7.7
   b) 0.05 M Tris buffer, pH 7.7
2. Adenosine deaminase is obtained from Sigma Chemical Co.

   Adenosine deaminase is added to 0.05 M Tris-HCl buffer, pH 7.7 for final resuspension of the membrane pellet, such that the concentration in the assay is 0.1 U/ml of tissue.
3. Cyclohexyladenosine, N^6-[Adenine-2,8-^{3}H] (specific activity 34 mCi/mmol) is obtained from New England Nuclear.

   For IC_{50} determinations: [^{3}H]CHA is made up to a concentration of 40 nM and 50 µl are added to each tube. This yields a final concentration of 1 nM in the assay.
4. Theophylline is obtained from Regis Chemical Co.

   A 100 mM stock solution is made up in deionized water. 20 µl are added to each of 3 tubes for the determination of nonspecific binding, yielding a 1 mM final concentration in the assay.
5. Test compounds

   For most assays, a 1 mM stock solution is prepared in DMSO and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue preparation

Male Wistar rats are sacrificed by decapitation. Whole brains minus cerebellum are removed, weighed and homogenized in 10 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48 000 *g* for 10 min, the supernatant decanted, the pellet resuspended in the same volume of buffer and centrifuged again as before. The final pellet is resuspended in 0.05 M Tris buffer containing 0.1 U/ml of adenosine deaminase.

Assay

1 000 µl	tissue suspension
930 µl	H_2O
20 µl	vehicle or theophylline or appropriate concentration of test compound
50 µl	^{3}H-CHA

The tubes are incubated for 2 hours at 25 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 3 times with 5 ml of 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml liquiscintillation cocktail, left to soak overnight and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 1 mM theophylline. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The complexity of interaction of adenosine ligands with receptors (Bruns et al. 1986) precludes the simple calculation of K_i values by the Cheng-Prusoff equation.

MODIFICATIONS OF THE METHOD

Stiles et al. (1985) used ^{125}I-labeled N^6-2-(4-aminophenyl)ethyladenosine as a selective ligand to probe the structure of A_1 receptors.

Lohse et al. (1987) described 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) as a high affinity antagonist radioligand for A_1 adenosine receptors.

Klotz et al. (1989) described 2-chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA) as a high affinity agonist radioligand for A_1 adenosine receptors.

Von Lubitz et al. (1995) studied the therapeutic implications of chronic NMDA receptor stimulation on adenosine A_1 receptors.

The partial agonism of theophylline-7-riboside on the adenosine A_1 receptor has been reported by Ijzerman et al. (1994).

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A.1.1.9 Adenosine A_2 receptor binding

PURPOSE AND RATIONALE

The A_2 receptor is a low-affinity binding site for adenosine (Daly et al. 1981). Activation of the A_2 receptor subtype by agonists mediates an increase in adenylate cyclase activity, while the A_1 receptor has the opposite effect. Although many of the physiological effects of adenosine seem to correlate with activity at the A_1 receptor, the effect on coronary blood flow correlates with activation of A_2 receptors (Hamilton et al. 1987).

This assay uses ^{3}H-NECA (5'-N-ethylcarboxamido [8-^{3}H]adenosine) to label A_2 receptors in rat striatum by the method described by Bruns et al. (1986). Comparison of data from this assay and the A_1 receptor assay provides a measure of selectivity for these two receptors.

PROCEDURE

Reagents

1. a) 0.5 M Tris buffer, pH 7.7
   b) 0.05 M Tris buffer, pH 7.7
   c) 0.05 M Tris buffer, pH 7.7, containing 12 mM $CaCl_2$ (final assay concentration: 10 mM)
2. Adenosine deaminase is obtained from Sigma Chemical Co.

   Adenosine deaminase is added to 0.05 M Tris-HCl buffer, pH 7.7, containing 12 mM $CaCl_2$ for final resuspension of the membrane pellet, such that the concentration in the assay is 0.1 U/ml of tissue.
3. 5'-N-Ethylcarboxamido[8-^{3}H]adenosine (specific activity 23–40 mCi/mmol) is obtained from Amersham.

   For IC_{50} determinations: ^{3}H-NECA is made up to a concentration of 80 nM and 50 µl is added to each tube. This yields a final concentration of 4 nM in the assay.
4. Cyclopentyladenosine (CPA) is obtained from Research Biochemicals Inc.

   A 5 mM stock solution is made up in DMSO. 20 µl are added to each of 3 tubes for the determination of nonspecific binding, yielding a 100 µM final concentration in the assay.

   Since [^{3}H]NECA is not a specific ligand for A_2 receptors, CPA is added to all other tubes to mask the A_1 receptors at a final concentration of 50 nM.
5. Test compounds

   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2×10^{-5} to 2×10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue preparation

Male Wistar rats are sacrificed by decapitation. Striata are removed, weighed and homogenized in 10 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48 000 *g* for 10 min, the supernatant decanted, the pellet resuspended in the same volume of buffer and centrifuged again as before. The final pellet is resuspended in 100 volumes of 0.05 M Tris buffer containing 10 mM $CaCl_2$ and 0.1 U/ml of adenosine deaminase.

Assay

830 µl	tissue suspension
100 µl	CPA
20 µl	vehicle or CPA or appropriate concentration of test compound
50 µl	^{3}H-NECA

The tubes are incubated at 25 °C for 2 hours. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 3 times with 5 ml of 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml Liquiscint scintillation cocktail, left to soak overnight and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 100 µM CPA. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The complexity of interaction of adenosine ligands with receptors precludes the simple calculation of K_i values by the Cheng-Prusoff equation.

MODIFICATIONS OF THE METHOD

Jarvis et al. (1989) reported on [^{3}H]CGS 21 680, a selective A_2 adenosine receptor agonist which directly labels A_2 receptors in rat brain. [^{3}H]CGS 21 680 binding was greatest in striatal membranes with negligible specific binding obtained in rat cortical membranes.

Gurden et al. (1993) described the functional characterization of three adenosine receptor types.

Hutchinson et al. (1990) described 2-(arylalkylamino)adenosin-5'-uronamides as a new class of highly selective adenosine A_2 receptor ligands.

A_{2A} Adenosine receptors from rat striatum and rat pheochromocytoma PC12 cells have been characterized with radioligand binding and by activation of adenylate cyclase (Hide et al1992).

Nonaka et al. (1994) reported on KF17837 ((E)-8-(3,4-dimethoxystyryl)-1,3-dipropyl-7-methylxantine), a potent and selective adenosine A_2 receptor antagonist.

The *in vitro* pharmacology of ZM 241385, a potent, non-xanthine, A_{2a} selective adenosine receptor antagonist has been reported by Poucher et al. (1955).

Monopoli et al. (1994) described the pharmacology of the selective $A_{2\alpha}$ adenosine receptor agonist 2-hexynyl-5'-N-ethylcarboxamidoadenosine.

Jacobson et al. (1993) described structure-activity relationships of 8-styrylxanthines as A_2-selective adenosine antagonists.

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A.1.1.10 Adenosine A_3 receptor binding

PURPOSE AND RATIONALE

The A_3 adenosine receptor has been cloned and characterized by Zhou et al. (1992). A possible role in reproduction has been discussed. The role of central A_3 adenosine receptors may be the mediation of behavioral depressant effects (Jacobson et al. 1993). The design of selective ligands of A_3 adenosine receptors and the therapeutic concepts including effects on locomotor activity, cardiovascular effects, effects in cerebral ischemia (von Lubitz et al. 1994), in cardiac preconditioning and as antagonists in inflammation and asthma has been discussed by Jacobson et al. (1995). Von Lubitz et al. (1995) noticed some anticonvulsive activity of the adenosine A_3 receptor selective agonist IB-MECA (N^6-(3-iodobenzyl) adenosine-5'-N-methyl-carboxamide). Stimulation of the A_3 adenosine receptor facilitates release of allergic mediators in mast cells (Ramkumar et al. 1993) inducing hypotension in the rat (Hannon et al. 1995). A binding site model and structure-activity relationships for the rat A_3 adenosine receptor are described by van Galen et al. (1994).

PROCEDURE

Cell culture and membrane preparation

Chinese hamster ovary (CHO) cells stably expressing the rat A_3 adenosine receptor are grown in F-12 medium containing 10% fetal bovine serum and penicillin/streptomycin (100 units/ml and 100 µg/ml, respectively) at 37° in a 5% CO_2 atmosphere. When cells reach confluency, they are washed twice with 10 ml of ice-cold lysis buffer (10 mM EDTA, pH 7.4). After addition of 5 ml of lysis buffer, cells are mechanically scraped and homogenized in an ice-cold Dounce homogenizer. The suspension is centrifuged at 43 000 *g* for 10 min. The pellet is suspended in the minimum volume of ice-cold 50 mM Tris/10 mM $MgCl_2$/1 mM EDTA (pH 8.26 at 5 °C) buffer required for the bind-

ing assay and homogenized in a Dounce homogenizer. Aminodeaminase (ADA, Boehringer Mannheim) is added to a final concentration of 3 units/ml and the suspension is incubated at 37 °C for 15 min; the membrane suspension is subsequently kept on ice until use.

Radioligand binding assay

Binding of [^{125}I]APNEA (N^6-2-(4-aminophenyl)ethyladenosine) to CHO cells stably transfected with the rat A_3 adenosine receptor clone is performed according to Stiles et al. (1985). Assays are performed in 50/10/1 buffer in glass tubes and contain 100 µl of the membrane suspension, 50 µl of inhibitor. Incubations are carried out in duplicate for 1 h at 37 °C and are terminated by rapid filtration over Whatman GF/B filters, using a Brandell cell harvester. Tubes are washed three times with 3 ml of buffer. Radioactivity is determined in a Beckman γ-counter. Non-specific binding is determined in the presence of 40 µM R-PIA=N^6-[(R)]-1-methyl-2-phenylethyl]adenosine.

EVALUATION

K_i values are calculated according to Cheng and Prusoff (1973), assuming a K_d for [^{125}I]APNEA of 17 nM.

MODIFICATIONS OF THE METHOD

^{125}I-4-aminobenzyl-5'-N-methylcarboxamidoadenosine has been recommended as a high affinity radio-ligand for the rat A_3 adenosine receptor (Olah et al. 1994).

Molecular cloning and functional expression of a sheep A_3 adenosine receptor has been reported by Linden et al. (1993).

G protein-dependent activation of phospholipase C by adenosine A_3 receptors in rat brain was reported by Abbracchio et al. (1995).

Molecular cloning and characterization of the human A_3 adenosine receptor was reported by Salvatore et al. (1993).

The differential interaction of the rat A_3 adenosine receptor with multiple G-proteins has been described by Palmer et al. (1995).

Baraldi and Borea (2000) described new potent and selective human adenosine A_3 receptor antagonists using radioligand binding studies to the human A_3 receptor.

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A.1.1.11 Inhibition of adenosine uptake in human erythrocytes

PURPOSE AND RATIONALE

Adenosine regulates multiple physiological functions in animals and humans. It plays a potent neuromodulatory role mainly by inhibiting the presynaptic transmitter release, e.g. of glutamate and aspartate. It is released by synaptic stimulation and during hypoxia in the central and peripheral nervous system. Adenosine

plays a neuroprotective role in hypoxia and ischemia since it reduces the excessive stimulation of the NMDA receptors. The use of adenosine uptake inhibitors has been proposed as a new therapeutic strategy for hypoxic/ischemic disease. Due to its vasodilatory action adenosine plays a key role in the regulation of coronary and cerebral blood flow. The rapid cellular uptake of adenosine by erythrocytes is a reason for the short duration of action of adenosine.

Human erythrocytes are used as a cellular model to detect adenosine uptake inhibitors. Erythrocytes are treated with test compound and thereafter incubated with ^{3}H-adenosine. The uptake of ^{3}H-adenosine is evaluated in relation to the untreated control group.

Dipyridamole is a potent inhibitor of adenosine uptake (IC_{50} of 3×10^{-7} M).

Standard compounds:
- theophylline
- dipyridamole (Persantin®)
- propentofylline (HWA 285)

PROCEDURE

Materials and solutions

isotonic glycyl-glycine buffer, pH 7.4

KCl	5.0 mM
NaCl	119.5 mM
$MgCl_2$	2.0 mM
glycyl-glycine	50 mM
Na_2HPO_4	2.0 mM
2-[^{3}H]-adenosine (specific activity 0.2 µCi/µmol)	5 µM

Buffer-washed fresh human erythrocytes are depleted of ATP by incubation in an isotonic glycyl-glycine buffer at 37 °C. Aliquots of the erythrocyte suspensions are incubated for 2 min in fresh glycyl-glycine buffer solution containing additional 10 mM glucose and test- or standard compound. In screening assays, test compounds are added at a concentration of 5×10^{-4} M. Drugs showing an effect in this assays, are further tested at a concentration range of 10^{-5}–5×10^{-4} M to determine IC_{50} values (triplicate samples for each concentration).

The suspension is then incubated with 5 µM radioactively labelled 2-[^{3}H]-adenosine for 30 s. The adenosine uptake is stopped by adding cold buffer (4 °C) containing 5 µM adenosine, 10 µM glucose and 7.4 µM dipyridamole. After centrifugation, the tritium radioactivity is determined in the supernatant.

EVALUATION

The percent change of ^{3}H-adenosine uptake relative to the vehicle control group is determined. The ^{3}H-adenosine uptake of the control group is taken as 100%; subsequent results are expressed as percentages of this.

IC_{50} values are determined by plotting the percent inhibition against test compound concentration; IC_{50} is defined as the dose of drug leading to a 50% inhibition of adenosine uptake.

Statistical evaluation is performed by means of the Student's *t*-test.

Standard data:
- IC_{50} of dipyridamole 3×10^{-7} M

MODIFICATIONS OF THE METHOD

Marangos et al. (1982), Verma and Marangos (1985) recommended [^{3}H]nitrobenzylthioinosine binding as a probe for the study of adenosine uptake sites in brain of various species. The highest density of binding sites were found in the caudate and hypothalamus of human and rat brain.

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A.1.1.12 Inhibition of the angiotensin-converting enzyme *in vitro*

GENERAL CONSIDERATIONS

Vasopeptidase inhibitors (VPIs) inhibit both angiotensinconverting enzyme (ACE) and neprilysin (NEP) and can thus reduce the activity of the renin-angiotensin system and potentiate the vasodilatory, natriuretic and antiproliferative effects of bradykinin and natriuretic peptides (Burnett 1999; Bralet and Schwartz 2001). Combined inhibition of neutral endopeptidase 24.11 (NEP) and angiotensin converting enzyme is a candidate therapy for hypertension and cardiac failure (Duncan et al. 1999).

PURPOSE AND RATIONALE

An *in vitro* system can be used to screen potential angiotensin-converting-enzyme inhibitors. Fluorescence generated by an artificial substrate in presence or absence of the inhibitor is measured to detect inhibitory activity.

PROCEDURE

Reagents

1. 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl
2. 10 mM potassium phosphate buffer, pH 8.3
3. Substrate: O-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (molecular weight 482) (Bachem Gentec. Inc., Torrance, California, USA)
   a) stock solution: 10 mg substrate in 10 ml 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl
   b) working solution: 2 ml stock solution is added to 18 ml 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl; final concentration in the assay is 170.2 µM.
4. Test compounds

   Compounds are made up to a concentration of 1 mM in 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl or 10% methanol in Tris/NaCl if insoluble in aqueous buffer alone. This will give a final concentration in the assay of 0.1 mM. If inhibition is seen, further dilution in Tris/NaCl should be made.

Enzyme preparation

Lung tissue from 10 rats is diced and homogenized in a blender with 3 pulses of 15 s each. The homogenate is centrifuged at 5 000 *g* for 10 min. The pellet is discarded, the supernatant is dialyzed against three 1 liter changes of 10 mM potassium phosphate buffer, pH 8.3 overnight in the cold and then centrifuged at 40 000 *g* for 20 min. The pellet is discarded, 390 mg $(NH_4)_2SO_4$ is added for each ml of supernatant. This will give 60% saturation. The solution is stirred on ice for 15 min. The pellet formed is dissolved in 15 ml potassium phosphate buffer, pH 8.3 and dialyzed against the same buffer overnight in the cold with three 1 liter changes. Some protein will precipitate during dialysis. The suspension is centrifuged at 40 000 *g* for 20 min and the supernatant is discarded. The final solubilized enzyme preparation can be aliquoted and stored at –20 °C at least 6 months.

Enzyme inhibition studies

1. Enzyme activity is measured with a Perkin Elmer LS-5 Fluorescence Spectrophotometer or equivalent at an excitation wavelength of 357 nm and an emission wavelength of 424 nm.
2. Enzyme assay

   50 µl vehicle or inhibitor solution and 40 µl enzyme are preincubated for 5 min, then 410 µl substrate working solution is added.

   Samples are mixed by drawing fluid back up into the pipette and by pipetting into the cuvette. For the initial control run of the day, the auto zero is pushed immediately after placing the sample in the cuvette.

EVALUATION

The individual fluorescence slope is measured and % inhibition is calculated as follows:

$$\%\ \text{inhibition} = 100 - \frac{\text{slope in presence of inhibitor}}{\text{control slope}} \times 100$$

Inhibitor concentrations on either side of the IC_{50} should be tested to generate a dose-response curve. The IC_{50} is calculated using Litchfield-Wilcoxon log-probit analysis.

Standard data:

- IC_{50} values for inhibition of angiotensin I-converting enzyme

– Compound	IC_{50} [M]
– Captopril	6.9×10^{-9}

MODIFICATIONS OF THE METHOD

Other assays use the cleavage of hippuric acid from tripeptides (Hip-Gly-Gly or Hip-His-Leu) whereby hippuric acid is either tritium labelled or determined spectrophotometrically (Cushman and Cheung 1969, 1971; Friedland and Silverstein 1976; Santos et al. 1985; Hecker et al. 1994).

Bünning (1984) studied the binding and inhibition kinetics of ramipril and ramiprilate (Hoe 498 diacid) with highly purified angiotensin converting enzyme using furanacryloyl-Phe-Gly-Gly as substrate.

The importance of tissue converting enzyme inhibition in addition to inhibition in plasma has been verified in several studies (Unger et al. 1984, 1985; Linz and Schölkens 1987).

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A.1.1.13 Quantitative autoradiographic localization of angiotensin-converting enzyme

PURPOSE AND RATIONALE

Cardiac angiotensin converting enzyme can be quantified in tissue, such as in rat hearts with chronic infarction after left coronary ligation, by computerized *in vitro* autoradiography (Kohzuki et al. 1996)

PROCEDURE

Myocardial infarction is induced in Wistar rats by left coronary artery ligation (see A.3.2.2). After various time intervals (1–8 months) the animals are decapitated, the hearts rapidly removed, and snap-frozen in isopentane at –40 °C. Frozen section (20 µm) are cut in a cryostat at –20 °C. The sections are thaw-mounted onto gelatin-coated slides, dried in a desiccator for 2 h at 4 °C and then stored at –80 °C.

Quantitative autoradiography

Radioligand: MK351A is a tyrosyl derivative of lisinopril, a potent competitive inhibitor of ACE. MK351A is iodinated by the chloramine T method and separated free from ^{125}I by SP Sephadex C25 column chromatography.

^{125}I-MK351A binding: The sections are preincubated in 10 mmol/L sodium phosphate buffer, pH 7.4, containing 150 mmol/L NaCl and 2% bovine serum albumin for 15 min at 20 °C. The sections are then incubated with 11.1 KBq/ml ^{125}I-MK351A in the same buffer for 60 min at 20 °C. Nonspecific binding is determined in the presence of 10^{-6} mol/L MK351A or lisinopril. Binding isotherms are determined using a set of serial sections incubated with 10^{-12}–10^{-6} mol/L lisinopril for 60 min.

After incubation, the sections are rapidly dried under a stream of cold air, placed in X-ray cassettes, and exposed to Agfa Scopix CR3 X-ray film for 12–72 h at room temperature. After exposure, the sections are fixed in formaldehyde and stained with haematoxylin and eosin. The optical density of the X-ray films is quantified using an imaging device controlled by a personal computer.

EVALUATION

The optical density of the autoradiographs is calibrated in terms of the radioactivity density in dpm/mm^2 with reference standards maintained through the procedure. The apparent binding site concentration (B_{max}) and binding affinity constant (K_A) in all the areas (excluding coronary arteries) of the right ventricle, intraventricular septum, the infarcted area in the left ventricle and the non-infarcted area in the left ventricle are estimated by an iterative non-linear model-fitting computer program LIGAND (Munson and Rodbard 1980).

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A.1.1.14 Angiotensin antagonism

The renin-angiotensin-aldosterone hormonal axis is the major long-term control for regulation of both arterial blood pressure and sodium balance. It supports normotension or hypertension via angiotensin vasoconstriction and angiotensin plus aldosterone-induced renal sodium retention (Laragh 1993).

Volpe et al. (1995) Wagner et al. (1996) showed that regulation of aldosterone biosynthesis by adrenal renin is mediated through AT_1 receptors in renin transgenic rats.

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A.1.1.14.1 Angiotensin II receptor binding

PURPOSE AND RATIONALE

Angiotensin II receptor subtypes, AT_1 and AT_2, have been identified by structurally dissimilar antagonists, by different distribution in organs of various species and with specific radioligands (Chiu et al. 1989, 1990, 1992, 1993; Chang and Lotti 1991; Gibson et al. 1991; Chansel et al. 1992; Steckelings et al. 1992; Aiyar et al. 1993; Barnes et al. 1993; Bossé et al. 1993; Bottari et al. 1993; Dzau et al. 1993; Feuillan et al. 1993; van Meel et al. 1993; Alexander et al. 2001). These two types of receptors have been cloned (Sasaki et al. 1991; Murphy et al. 1991; Mukoyama et al. 1993; Kambayashi et al. 1993). Two other mammalian receptors named AT_3 and AT_4 have been described (de Gasparo et al. 1998).

The functional correlates of angiotensin II receptors have been discussed by Timmermans et al. (1992, 1993; Bernstein and Berk 1993). Most effects of angiotensin are mediated via the AT_1 receptors, but a possible role of angiotensin II subtype AT_2 receptors in endothelial cells and isolated ischemic rat hearts has been suggested (Wiemer et al. 1993a,b). Clearance studies in dogs indicated that the angiotensin type 2 receptor may be related to water handling in the kidney (Keiser et al. 1992).

Evidence for AT_1 receptor subtypes (AT_{1A} and AT_{1B}) has been reported (Iwai and Inagami 1992; Kakar et al. 1992; Balmforth et al. 1994; Matsubara et al. 1994; Bauer and Reams 1995; de Gasparo et al. 1998).

The assay described below is used to determine the affinity of test compounds to the angiotensin II receptor by measuring their inhibitory activity on the binding of ^{3}H-angiotensin II to a plasma membrane preparation from rat or bovine adrenal cortex.

PROCEDURE

Fresh bovine adrenal glands are obtained from the local slaughter house. For rat adrenal glands, male Sprague-Dawley rats weighing 250–300 g are sacrificed. The adrenals are separated from fat tissue and the medullae removed. The cortices are minced and homogenized in 5 mM Tris buffer containing 1 mM $MgCl_2$ and 250 mM sucrose, pH 7.4, using a chilled Potter homogenizer. The homogenate is centrifuged at 3 000 *g* and 4 °C for 10 min. The supernatant is recentrifuged at 39 000 *g* and 4 °C for 10 min. The pellets are resuspended in 75 mM Tris buffer containing 25 mM $MgCl_2$, pH 7.4, and recentrifuged twice at 39 000 *g* and 4 °C for 10 min. After the last centrifugation, the pellets are suspended in 75 mM Tris buffer containing 25 mM $MgCl_2$ and 250 mM sucrose, pH 7.4. Samples of 0.5 ml are frozen in liquid nitrogen and stored at –70 °C.

In the competition experiment, 50 µl ^{3}H-angiotensin II (one constant concentration of $0.5–1\times10^{-9}$ M), and 50 µl test compound (6 concentrations, $10^{-5}–10^{-10}$ M) and 100 µl membrane suspension from rat or bovine adrenal cortex (approx. 250 mg wet weight/ml) per sample are incubated in a bath shaker at 25 °C for 60 min. The incubation buffer contains 50 mM HEPES, 0.1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$ and 0.2% bovine serum albumin, pH 7.4.

Saturation experiments are performed with 12 concentrations of ^{3}H-angiotensin II ($15–0.007\times10^{-9}$ M). Total binding is determined in the presence of incubation buffer, non-specific binding is determined in the presence of non-labeled angiotensin II (10^{-6} M).

The reaction is stopped by rapid vacuum filtration through glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free one. The retained membrane-bound radioactivity on the filter is measured after addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

EVALUATION OF RESULTS

The following parameters are calculated:

- total binding of ^{3}H-angiotensin II
- non-specific binding: binding of ^{3}H-angiotensin II in the presence of mepyramine or doxepine
- specific binding = total binding – non-specific binding
- % inhibition of ^{3}H-angiotensin II binding: 100 – specific binding as percentage of control value

The dissociation constant (K_i) and the IC_{50} value of the test drug are determined from the competition experiment of ^{3}H-angiotensin II versus non-labeled drug by a computer-supported analysis of the binding data (McPherson 1985).

MODIFICATIONS OF THE METHOD

Olins et al. (1993) performed competition studies in rat uterine smooth muscle membranes and rat adrenal cortex membranes using [^{125}I] labeled angiotensin II.

Membranes from cultured rat aortic smooth muscle cells and from human myometrium were used for binding studies with [^{125}I] labeled angiotensin II by Criscone et al. (1993).

Wiener et al. (1993) used membrane preparations from rat lung and adrenal medulla for binding studies with [^{125}I] labeled angiotensin II.

Bradbury et al. (1993) used a guinea pig adrenal membrane preparation to study nonpeptide angiotensin II receptor antagonists.

Cazaubon et al. (1993) prepared purified plasma membranes from rat livers for [^{125}I] AII binding assays.

Kushida et al. (1995) tested AT II receptor binding in particulate fractions of rat mesenteric artery and rat adrenal cortex and medulla with ^{125}I-AT II.

Chang et al. (1995) used rabbit aorta, rat adrenal and human AT_1 receptors in CHO cells and AT_2 receptors from rat adrenal and brain to characterize a nonpeptide angiotensin antagonist.

Aiyar et al. (1995) tested inhibition of [^{125}I]angiotensin II or [^{125}I]angiotensin II (Sar^1,Ile^8) binding in various membrane and cell preparations, such as rat mesenteric artery, rat adrenal cortex, rat aortic smooth muscle cell, human liver, recombinant human AT_1 receptor, bovine cerebellum, and bovine ovary.

Caussade et al. (1995) tested [^{125}I]Sar^1,Ile^8-angiotensin II binding to rat adrenal membranes and rat aortic smooth muscle cells.

Using [^{125}I]Sar^1,Ile^8-angiotensin II as radioligand, de Gasparo and Whitebread (1995) compared the affinity constants of valsartan and losartan in liver and adrenal of rat and marmoset, human adrenal and in rat aortic smooth muscle cells.

Kiyama et al. (1995) used COS cells transfected with a cDNA encoding a human AT_1 angiotensin II receptor to evaluate nonpeptide angiotensin II receptor antagonists.

Mizuno et al. (1995) used bovine adrenal cortical membranes, Nozawa et al. (1997) membrane fractions from rat aorta, bovine cerebellum and human myocardium and [^{125}I]angiotensin II as radioligand.

Renzetti et al. (1995a,b) used membranes from rat adrenal cortex and bovine cerebellum for binding assays with [^{3}H]angiotensin II as radioligand.

Inter-species differences in angiotensin AT_1 receptors were investigated by Kawano et al. (1998).

The angiotensin II receptor subtype having a high affinity for lorsatan has been designated angiotensin AT_1 receptor and the receptor having a high affinity for PD123177 (1-(3-methyl-4-aminophenyl) methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1H-imidazo[3,5-c]pyridine-6-carboxylic acid) as angiotensin AT_2 receptor (Bumpus et al. 1991; Nozawa et al. 1994; Chang et al. 1995).

In order to determine affinity for the angiotensin AT_1 subtype in a radioligand binding assay with ^{125}I-sarcosine^1, isoleucine^8 angiotensin°II, Chang and Lotti (1991), Chang et al. 1995), Wong et al. (1995) incubated membranes of tissues with both AT_1 and AT_2 receptors in the presence of 1 µM PD121981 (which occupied all the AT_2 binding sites) and for the angiotensin AT_2 subtype in the presence of 1 µM lorsatan (which occupied all the AT_1 binding sites).

Hilditch et al. (1995) used membranes from rat livers and [^{3}H]-AT II for the determination of binding affinity at AT_1 receptors, or membranes from bovine cerebellum and [^{125}I]-Tyr^4-AT II for AT_2 receptors.

Lu et al. (1995) studied the influence of freezing on the binding of ^{125}I-sarcosine^1, isoleucine^8 angiotensin°II to angiotensin°II receptor subtypes in the rat. The results suggested that studies of AII receptor subtypes that involve freezing of the tissue underestimate the density and affinity of the AT_1 receptor subtype.

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A.1.1.14.2 Angiotensin II induced contraction in isolated rabbit aorta

PURPOSE AND RATIONALE

The isolated rabbit aorta has been used to evaluate angiotensin II agonists (Liu 1993) and angiotensin II antagonists (Chang et al. 1992, 1994; Noda et al. 1993; Aiyar et al. 1995; Cirillo et al. 1995; Kushida et al. 1995; Mochizuki et al. 1995; Renzetti et al. 1995; Wong et al. 1995; Hong et al. 1998; Kawano et al. 1998).

PROCEDURE

New Zealand White male rabbits weighing 2–3 kg are sacrificed and exsanguinated. The thoracic aorta is removed and cleaned from adherent fat and connective tissue. The vascular endothelium is removed by gently rubbing the intimal surface of the vessel. Spiral aortic strips (2–3 mm wide and 30 mm long) are prepared and mounted in 5 ml organ baths containing Krebs-Henseleit solution (120 mM NaCl, 4.7 mM KCl, 4.7 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, glucose 10 mM, pH 7.4). The organ baths are kept at 37 °C and gassed continuously with 95% O_2 / 5% CO_2. Strips are attached to isometric transducers connected to a polygraph and a resting tension of 1 g is applied to each strip. Changes in contraction are analyzed with a digital computer. Aortic strips are allowed to equilibrate for 1 h and washed every 15 min. Two consecutive contractile-response curves to cumulative addition of ATII (0.1–300 mM) are constructed. After each curve the strips are washed 4 times and allowed to relax to the baseline tension. Afterward, each strip is incubated for 30 min with the vehicle or with a single concentration of the antagonist (1 – 10 – 100 – 1 000 mM) before a third concentration-response curve to angiotensin II is obtained.

EVALUATION

The result of each concentration is expressed as a percentage of maximum response to AII. The pA_2 and pD'_2 values are calculated (van Rossum 1963).

MODIFICATIONS OF THE METHOD

Isolated guinea pig aortas were used by Mizuno et al. (1995).

Cirillo et al. (1995) evaluated the antagonism against AII-induced vasoconstriction in rat isolated perfused kidney.

Chang et al. (1992, 1994) determined AII-induced aldosterone release in rat adrenal cells and AII-induced [^{3}H]inositol phosphate accumulation in cultured rat aorta smooth muscle cells.

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A.1.1.14.3 Angiotensin II antagonism *in vivo*

PURPOSE AND RATIONALE

The effect of ATII antagonists on blood pressure has been measured in anesthetized (Olins et al. 1993; Beauchamp et al. 1995; Kawano et al. 1998), in pithed (Cazes et al. 1995; Christophe et al. 1995; Cirillo et al. 1995; Deprez et al. 1995; Häuser et al. 1998) and in conscious (Junggren et al. 1996; Nozawa et al. 1997; Shibasaki et al. 1997; Hashimoto et al. 1998) normotensive and hypertensive rats.

PROCEDURE

Male Sprague-Dawley rats are anesthetized with 100 mg/kg i.p. Inactin® and placed on servo-controlled heating pads to maintain body temperature between 37 °C and 38 °C. PE50 catheters are implanted in the femoral artery and vein to measure arterial blood pressure and administer compounds, respectively. A catheter is placed in the trachea to ensure airway patency. Arterial pressure is measured continuously by connecting the arterial catheter to transducer coupled to a Gould pressure transducer. The output is recorded on a polygraph. Mean arterial pressure is derived electronically. After a 30–45 min stabilization period, autonomic transmission is blocked by treatment with mecamylamine (3 mg/kg i.v.) and atropine (0.4 mg/kg i.v.). After arterial pressure has stabilized, angiotensin is infused i.v. in isotonic saline with a syringe pump. When the pressure response to angiotensin has stabilized, angiotensin II antagonists are given in increasing doses. The doses are given intravenously in a cumulative fashion, i.e., the next highest dose is given at the time of maximum response to the prior dose.

EVALUATION

Data are presented as percent inhibition of the angiotensin pressor response to each dose of the antagonists and plotted against the log of the cumulative doses of antagonist. Linear regression is used to calculate the dose at which the response to angiotensin is inhibited 50% (ID_{50}) for each rat. Means ±SEM are calculated.

MODIFICATIONS OF THE METHOD

Olins et al. (1993), Cirillo et al. (1995) determined also the antihypertensive effects in conscious spontaneously hypertensive rats and in conscious sodium-deficient dogs.

Stasch et al. (1997) studied the long-term blockade of the angiotensin II receptor in renin trangenic rats, salt-loaded Dahl rats, and stroke-prone spontaneously hypertensive rats.

Nishioka et al. (1998), Richter et al. (1998) used the (mRen-2)27 transgenic (Tg^+) rat, a hypertensive model dependent on increased expression of the renin angiotensin system, to explore the role of angiotensin AT_2 receptors in the control of cardiovascular and renal excretory function.

Simoes e Silva et al. (1998) evaluated the effects of chronic administration of an angiotensin antagonist on diuresis and natriuresis in normotensive and spontaneously hypertensive rats.

Kai et al. (1998) examined the effects of an angiotensin II type 1 antagonist on cardiac hypertrophy and nephropathy using Tsukuba hypertensive mice (THM) carrying both human renin and angiotensinogen genes.

Kivlighn et al. (1995a,b), Gabel et al. (1995), studied angiotensin II antagonists in conscious rats, dogs, rhesus monkeys and chimpancees.

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Yamamoto et al. (1997), Ogilvie et al. (1998), studied angiotensin II receptor antagonists in acute heart failure induced by coronary artery ligation in anesthetized dogs and in chronic heart failure induced by left ventricular rapid-pacing in conscious dogs.

Massart et al. (1998) evaluated the cumulative hypotensive effects of angiotensin II- and endothelin-1-receptor antagonists in a model renovascular hypertension in dogs.

Hayashi et al. (1997) examined the hemodynamic effects of an angiotensin II type 1 receptor antagonist in rats with myocardial infarction induced by coronary ligation.

Kivlighn et al. (1995c) studied the effects of a nonpeptide that mimics the biological actions of angiotensin II in anesthetized rats.

Huckle et al. (1996) evaluated angiotensin II receptor antagonists for their ability to inhibit vascular intimal thickening in a porcine coronary artery model of vascular injury.

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A.1.1.15
Renin-inhibitory activity using human kidney renin and a synthetic substrate

PURPOSE AND RATIONALE

In contrast to other enzymes, renin shows a rather high species specificity. To be relevant for humans human renin has to be used. One of the reasons that human renin is specific for human angiotensinogen lies in the sequence of human angiotensinogen itself. Inhibition of renin is measured by angiotensinogen formed in the presence of angiotensinase inhibitors. The following procedure is used to determine the effect of potential renin inhibitors on purified human kidney renin without interference from plasma proteins or lipids.

PROCEDURE

The synthetic substrate represents the first fourteen amino acids of the N-terminus of human angiotensinogen: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asn. The assay mixture is composed of phosphate buffer (pH 7.5), bovine serum albumin, 3 mM EDTA, 0.01 mM phenylmethyl-sulfonyl fluoride (PMSF), 0.002% Genapol PF 10®, test compound (dissolved in DMSO), substrate (3 µM) and purified human kidney renin (Calbiochem GmbH, Frankfurt/M., Germany; cat. no. 553861). The mixture is incubated for two hours at 37 °C. Then the reaction is stopped by transfer of 450 µl into preheated (95 °C) Eppendorf® tubes. The amount of angiotensin I liberated is measured by RIA (Renin MAIA® kit, Serono Diagnostika GmbH, Freiburg, Germany).

Human angiotensinogen (0.2 µM) may be used as a substrate instead of the tetradekapeptide. The pH value of the incubation mixture may be lowered to 6.0 by using a maleic acid buffer; this results in higher renin activity. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) may be substituted for phosphate in the pH 7.5 buffer.

EVALUATION

Renin activity, i.e. angiotensin I production (ng/ml × 2 h), is corrected for an angiotensin I – like immunoreactivity which can be measured in the assay samples even in the absence of added renin. IC_{50} values are determined from a plot of renin activity (as per cent of control) vs. molar concentration of the test compound.

MODIFICATIONS OF THE METHOD

Wang et al. (1993) described a continuous fluorescence assay of renin activity employing a new fluorigenic peptide substrate.

Inhibition of plasma renin activity in blood samples from various species can be determined in order to evaluate the species specificity of a renin inhibitor (Linz et al. 1994)

Blood samples are obtained from dogs, sheep and rhesus monkeys by venipuncture. Wistar rats and guinea pigs are anesthetized with Nembutal® (60 mg/kg intraperitoneally) and the blood is collected by puncture of the abdominal aorta. Human blood is collected from volunteers (Donafix blood collecting set, Braun Melsungen AG, Melsungen, FR Germany) in cooled bottles. All blood samples are anticoagulated with Na-EDTA (final concentration 10–15 mM). The renin is dissolved in DMSO as 10^{-2} M stock solution and diluted before each experiment in DMSO. The endogenous formation of ANG I in plasma during incubation at 37 °C is determined as the measure of renin activity. Generation and quantitation of ANG I are performed using a commercial radio immunoassay kit (Renin-MAIA®, Serono Diagnostika GmbH, Freiburg, FR Germany). Plasma samples are thawed on ice and centrifuged after addition of 100 µl PMSF solution (kit) per 10 ml. The assay mixture contains 450 µl plasma plus 1% (v/v) PMSF solution, 45 µl buffer (phosphate buffer, pH = 7.4, + 10^{-5} M ramiprilate) and 5 µl renin inhibitor solution (diluted in DMSO as required) or pure DMSO for controls. The assay is incubated for an appropriate time (2–3 hours) at 37 °C. ANG I is measured in 100 µl samples (triplicate determinations). Basal ANG I immunoreactivity of the plasma is determined from an unincubated control assay (0 °C). This pre-incubation value is subtracted from all measurements. The renin activity in the presence of the renin inhibitor is calculated as percent activity in relation to control samples containing only DMSO. The IC_{50} value is determined from a semilogarithmic plot of percent renin activity versus concentration of the renin inhibitor.

Wood et al. (1990) determined the activity of a synthetic renin inhibitor against rat, mouse, dog, guinea pig, rabbit, cat, marmoset and human renin using plasma pools from these species. Plasma from each species was collected using EDTA as an anticoagulant. Samples of plasma were incubated at 37 °C in the presence or absence of varying concentrations of test compound. The ANG I formed was measured by radioimmunoassay.

Shibasaki et al. (1991) used squirrel monkeys to study the *in vivo* activity of a specific renin inhibitor after intravenous and oral application.

Bohlender et al. (1996) reconstructed the human renin-angiotensin system in transgenic rats overexpressing the human angiotensin gene TGR(hOGEN) 1623 by chronically injecting human recombinant renin intravenously using Alzet pumps.

Salimbeni et al. (1996) tested the *in vitro* inhibition of human plasma renin activity by two synthetic angiotensinogen transition state analogues.

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A.1.1.16 PAF binding assay

PRINCIPLE AND RATIONALE

Injection of platelet activating (PAF) factor induces a wide range of potent and specific effects on target cells, including aggregation of platelets and shock symptoms like systemic hypotension, pulmonary hypertension, increased vascular permeability, neutropenia and thrombocytopenia. Inhalation of PAF causes immediate bronchoconstriction followed by inflammation of the airways (further information see Sect. B.1.9).

The PAF receptor belongs to the superfamily of G protein-coupled receptors (Chao and Olson 1993, Izumi and Shimizu 1995). Cloning studies have indicated a single human PAF receptor gene containing an intron at the 5' flanking region, providing alternative sequences (Ishi and Shimizu 2000).

The following procedure is used to detect compounds that inhibit binding of ^{3}H-PAF (platelet activating factor) in rabbit platelets (PAF receptor).

PROCEDURE

Crude rabbit platelets are incubated in plastic tubes for 15 min at 25 °C in a buffer solution (0.54 g/l KH_2PO_4, 0.6 g/l Na_2HPO_4, 5.8 g/l NaCl, 1.0 g/l BSA, pH 7.1) with 1 nM synthetic ^{3}H-labeled PAF (1-*O*-[1,2-$^3H_2$]alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) and various concentrations of test compound. Non-specific binding is determined in the presence of 10 µM CV 3988. Bound ligand is separated from the incubation medium by rapid filtration through Whatman GF/C glass fibre filters. Following rinsing with ice-cold buffer (3 × 5 ml), the filters are placed in 10 ml scintillation cocktail for radioactivity determination.

EVALUATION

The following parameters are calculated:

- total binding of ^{3}H-PAF
- non-specific binding in the presence of 10 µM CV 3988
- specific binding = total binding – non-specific binding
- % inhibition: 100 – specific binding as percentage of the control value

Compounds are first tested at a single high concentration (5 000 nM) in triplicate. For those showing more than 50% inhibition a displacement curve is constructed using 7 different concentrations of test compound. Binding potency of compounds is expressed either as a "relative binding affinity" (RBA) with respect to the standard compound (CV 3988) which is tested in parallel or as an IC_{50}.

$$RBA = \frac{IC_{50}\ \text{standard compound}}{IC_{50}\ \text{compound}} \times 100\%$$

Standard data:

- CV 3988 IC_{50}: 276 nM ±24 ($n = 20$)

MODIFICATIONS OF THE METHOD

Several authors (Casals-Stenzel et al. 1987; Dent et al. 1989a,b; Ring et al. 1992; Ukena et al. 1988) used the specific platelet activating factor receptor antagonist [^{3}H]WEB-2086 or [^{3}H]Apafant to identify and characterize the PAF-receptors expressed on the cell surface of platelets, macrophages, and eosinophils.

Balsa et al. (1996) characterized [^{3}H]Apafant binding to the PAF receptor on rabbit platelet membranes and compared a microplate filtration system with the standard method.

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A.1.1.17 Endothelin

A.1.1.17.1 General considerations

Endothelin is a endothelium-derived peptide family consisting of three peptides (ET-1, ET-2, and ET-3) with very potent and long-lasting vasoconstrictive activity (Yanagisawa et al. 1988a,b; King et al. 1989; Miller et al. 1989; Yanagisawa and Masaki 1989; Inoue et al. 1989; Shinmi et al. 1989; Vanhoutte et al. 1992).

ET-1 is processed from prepro ET-1, pro-ET-1 to big ET-1, which is converted to ET-1 by the endothelin-converting-enzyme (ECE).

Subtypes of endothelin receptors have been described (Takayanagi et al. 1991; Miyazaki et al. 1992).

Molecular characterization of the ET_A and ET_B receptors was reported by Miyazaki et al. (1992), Sakuarai et al. (1992).

In addition, the existence of a third type, ET_C, was found in *Xenopus laevis* (Karne et al. 1993).

The comparison of recombinant endothelin receptors shows different affinity rank orders to the three endothelins (Masaki et al. 1994).

Grant et al. (1997) reported the *in vitro* expression of endothelin-1 (ET-1) and the ET_A and ET_B ET receptors by prostatic epithelium and stroma.

The ET peptides not only elicit potent and long-lasting contractions of isolated strips of various blood vessels *in vitro* but also increase blood pressure *in vivo* suggesting that this peptide family may be involved in the pathogenesis of cardiovascular diseases (Simonson and Dunn 1990; Masaki 1991; Doherty 1992; Goto et al. 1996; Gray and Webb 1996; Douglas and Ohlstein 1997).

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A.1.1.17.2 Evaluation of endothelin activity

PURPOSE AND RATIONALE

Most investigators used isolated arteries to evaluate the activity of endothelins and derivatives. Rodman et al. (1989) compared the potency and efficacy of porcine and rat endothelin in rat aortic and pulmonary rings.

PROCEDURE

Arterial rings are obtained from male Sprague Dawley rats weighing from 300–400 g. Rats are anesthetized with 50 mg/kg i.p. pentobarbital, the chest is opened, 100 units heparin sulfate are injected into the right ventricle, and the rats are exsanguinated. Rings are then isolated from either the descending thoracic aorta or the right main pulmonary artery, cleaned of adventitia, and suspended from Grass FT03 force-displacement transducers in muscle baths containing 10 ml of physiologic salt solution of the following composition ($\times 10^{-3}$ M): $CaCl_2$ 1.80, $MgSO_4$ 0.83, KCl 5.3.6, NaCl 116.34, NaH_2PO_4 0.40, D-glucose 5.50, and $NaHCO_3$ 10.04. The solution is maintained at 37 °C and bubbled with 21% O_2 and 5% CO_2. Endothelium-denuded rings are prepared by gently rubbing the intima with a roughened steel rod. Denudation is confirmed by the absence of relaxation to 10^{-5} M acetylcholine in rings precontracted with 10^{-7} M norepinephrine. Resting force is adjusted to the optimum resting tension of 0.75 g for pulmonary artery rings and 1.0 g for aortic rings. Maximum contraction to 8×10^{-2} M KCl is determined and subsequent responses to endothelin are expressed as a percentage of maximum KCl contraction for determination of maximum effectiveness or as a percentage of maximum endothelin contraction for determination of potency.

EVALUATION

Concentration-response curves are compared using the method of Carpenter (1986). Data are expressed as means ±SEM and statistical comparisons are performed using Student's *t*-test, with $P < 0.05$ considered significant.

MODIFICATIONS OF THE METHOD

Lembeck et al. (1989) studied the effects of endothelin on the cardiovascular system and on smooth muscle preparations in different species.

Reynolds and Mok (1990) studied the role of thromboxane A_2/prostaglandin H_2 receptor in the vasoconstrictor response of **rat aorta** to endothelin.

Pang et al. (1990) studied the cellular mechanisms of action of endothelin in **isolated canine coronary arteries**.

Lüscher et al. (1992) used **perfused and pressurized mesenteric resistance arteries of rats** and **human internal mammary arteries** to study the interaction between endothelin and endothelium-derived relaxing factors.

Advenier et al. (1990) studied the contractile activity of three endothelins (ET-1, ET-2 and ET-3) on the **human isolated bronchus**.

Wallace et al. (1989) compared the effects of endothelin-1 and endothelin-3 on the **rat stomach**.

Aldosterone secretion in cultured calf zona glomerulosa cells was stimulated by ET-1 and sarafotoxin S6b to a similar degree, but less than by angiotensin II (Gomez-Sanchez 1990).

Brock and Danthuluri (1992) used **cultured vascular smooth muscle cells** to study the cellular actions of endothelin.

Pigment dispersion in cultured dermal melanophores from *Xenopus laevis* was used as indicator of ET_C receptor mediated responses (Karne et al. 1993).

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A.1.1.17.3 Endothelin receptor antagonism *in vitro*

PURPOSE AND RATIONALE

Competitive endothelin antagonists are of therapeutic interest (Ihara et al. 1991; Fujimoto et al. 1992; Fukuroda et al. 1992; Urade et al. 1992; Breu et al. 1993; Mihara and Fujimoto 1993; Sogabe et al. 1993; Warner 1994; Opgenorth 1995; Brunner 1998).

A sensitive sandwich-enzyme immunoassay for human endothelin has been established by Suzuki et al. (1989).

PROCEDURE

The ventricles of rat hearts are minced with scissors and homogenized in 7 vol of ice-cold 20 mM $NaHCO_3$ containing 0.1 mM PMSF (Phenylmethylsulfonyl fluoride), pH 7.4, with a Polytron homogenizer (Brinkman Instruments Inc., Westberg, NY). The homogenates are centrifuged at 1 000 *g* for 10 min, and then the pellet discarded. The supernatant is centrifuged at 30 000 *g* for 30 min. The pellet is washed once and resuspended in Tris buffer (50 mM, pH 7.4 at 25 °C) containing 0.1 mM PMSF, and stored at –80 °C until use.

For binding studies (Gu et al. 1989) cardiac membranes (0.21 mg/ml as protein) are incubated with 25 pM [^{125}I]ET-1 or [^{125}I]ET-3 (New England Nuclear) in a final assay volume of 0.1 ml in borosilicated glass tubes, containing 50 mM Tris-HCl, 0.1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 250 µg/ml bacitracin, and 10 µg/ml soybean trypsin inhibitor (pH 7.4). Binding is performed for 60 min at 37 °C. The binding reaction is terminated by the addition of 2.5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), followed by a rapid filtration through a Whatman GF/C glass fibre filter (pre-soaked in 1% polyethylene-imine) under reduced pressure. The filters are then quickly washed 4 times with 2.5 ml of the buffer. Radioactivity retained on the filter is counted.

EVALUATION

Non-specific binding is defined in the presence of ET-1. Specific binding is the difference between total and non-specific binding. K_i-values and Scatchard plots are calculated.

MODIFICATIONS OF THE METHOD

The nomenclature of endothelin receptors has been reviewed by Alexander et al. (2001).

Functional endothelin/sarafotoxin receptors were described in rat heart myocytes (Galron et al. 1989) and in the rat uterus (Bousso-Mittler 1989).

Mihara and Fujimoto (1993) cultured rat aortic smooth muscle A7r5 cells expressing ET_A receptors (Takuwa et al. 1990) and human Girardi heart cells ex-pressing ET_B receptors (Mihara and Fujimoto 1992). Receptor specificity could be demonstrated.

Aramori et al. (1993) studied the receptor-binding properties and the antagonistic activities of an endothelin antagonist in transfected Chinese ovary hamster cells permanently expressing the two ET receptor subtypes (ET_A and ET_B).

De Juan et al. (1993) characterized an endothelin receptor subtype B in the retina of rats.

Clozel et al. (1994) performed binding assay on cells or membranes from baculovirus infected insect cells that expressed recombinant ET_A or ET_B receptor, CHO cells that expressed recombinant ET_A or ET_B receptor, cultured human vascular smooth muscle cells from umbilical veins, rat mesangial cells (for ET_A), microsomal membranes from human placenta and from porcine cerebellum (for ET_{B1}) and from porcine trachea (for ET_{B2}, using BQ-3020 or sarafotoxin S6C as ligand).

Williams et al. (1995) used CHO cells expressing cloned ET_A or ET_B receptors directly in binding and functional assays without preparing membranes from them.

Reynolds et al. (1995) used CHO-K1 cells expressing recombinant human ET_B receptor, Ltk^- cells expressing human ET_A receptor and rabbit renal artery vascular smooth muscle cells expressing rabbit ET_A receptor for evaluation of an ET_A receptor antagonist.

Rat or bovine cerebella were used for differentiation of receptor subtypes (Williams et al. 1991).

Peter and Davenport (1995) proposed a selective ligand for ET_A receptors.

Ihara et al. (1992), Watakabe et al. (1992) described radioligands for endothelin (ET_B) receptors.

Vigne et al. (1996) described the properties of an endothelin-3-sensitive eta-like endothelin receptor in brain capillary endothelial cells.

Stables et al. (1997) described a bioluminescent assay for agonist activity at G-protein-coupled receptors, such as the endothelin ET_A receptor. Transient expression of apoaequorin in CHO cells and reconstitution with the cofactor coelenterazine resulted in a large, concentration dependent agonist-mediated luminescent response following cotransfection with the endothelin ET_A, angiotensin AT_{II}, TRH and neurokinin NK_1 receptors, all of which interact predominantly with the $G\alpha_q$-like phosphoinositidase-linked G-proteins.

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A.1.1.17.4 Endothelin receptor antagonism *in vivo*

PURPOSE AND RATIONALE

Various pharmacological models have been used for the characterization of endothelins and endothelin antagonists, such as the isolated porcine coronary artery. (Hickey et al. 1985; Yanagisawa et al. 1988, 1989; Inoue et al. 1989; Kimura et al. 1989; Ihara et al. 1991; Fukuroda et al. 1991).

Since the smooth musculature is considered to contain mainly ET_A receptors the preparation is used to test ET_A antagonists.

PROCEDURE

Left anterior descending coronary arteries are isolated from fresh porcine hearts. Connective tissues and adherent fat are removed. For removal of vascular endothelium, the intimal surface of spiral strips is rubbed gently with filter paper. The endothelium-denuded arteries are

cut into spiral strips about 10 mm long and 1 mm wide. Each strip is suspended in an organ bath containing Krebs-Henseleit solution bubbled with 95% O_2 / 5% CO_2 at 37 °C. After equilibration, reference contraction is isometrically obtained with 50 mM KCl. Concentration-response curves for ET-1 are obtained by cumulative additions of ET-1. Antagonists are added 20 min before the cumulative additions of ET-1.

EVALUATION

The pA_2 values and slopes are obtained by analysis of Schild plots.

MODIFICATIONS OF THE METHOD

Calo et al. (1996) investigated three **rabbit vessels, the carotid, the pulmonary artery, and the jugular vein** to identify vascular monoreceptor systems, either ET(A) or ET(B), for structure-activity studies of endothelins and their antagonists.

Vedernikov et al. (1993) used **rings of the left circumflex coronary artery** from **dogs** which were denuded of endothelium and exposed to anoxic periods, Urade et al. (1992) **rat aortic smooth muscle denuded of the epithelium** and Sogabe et al. (1993) **spirally cut strips of rabbit aorta**.

Williams et al. (1995) used **rat aorta, rabbit iliac and pulmonary artery** for contractile assays, and **anesthetized ferrets and conscious normotensive dogs** as *in vivo* models to characterize a nonpeptidyl endothelin antagonist.

Itoh et al. (1993) studied the preventive effect of an ET_A receptor antagonist on **experimental cerebral vasospasm in dogs** using a two-hemorrhage model of subarachnoid hemorrhage. Clozel et al. (1993) performed similar experiments in rats.

The **vasodilating** effect in the **isolated perfused rat mesentery** which is found after infusion of rat endothelin (Warner et al. 1989) and after the selective ET_B receptor agonist sarafotoxin S6c (Williams et al. 1991) can be antagonized by an endothelin receptor antagonist (Clozel et al. 1993).

Ercan et al. (1996) found an increase of digoxin-induced ectopic ventricular complexes by endothelin peptides in **isolated guinea pig hearts**, which could be antagonized by an endothelin-A receptor antagonist.

The **endothelin-induced sustained increase of blood pressure** in anesthetized rats was studied by Yanagisawa et al. (1988), Inoue et al. (1989), Ihara et al. (1991). Intravenous bolus injection of endothelin causes a bi-phasic blood pressure response: a transient decrease, probably mediated from the release of vasodilator mediators (prostacyclin and EDRF), and a sustained increase (Rubanyi and Bothelho 1991).

Nishikibe et al. (1993) examined the **antihypertensive effect** of an endothelin antagonist in a genetic hypertensive model (**stroke-prone spontaneously hypertensive rats**).

Watanabe et al. (1995) characterized the pharmacological profile of a non-selective endothelin receptor antagonist and studied the **inhibition of myocardial infarct size** in rats.

The contractile activity of the **isolated guinea pig trachea without epithelium** and of the guinea pig longi-tudinal muscle was used by Urade et al. (1992) for determination of **ET_B receptor** mediated responses.

Spinella et al. (1991) assessed bioactivity of a specific endothelin-1 antagonist in an **isolated perfused guinea pig lung** preparation in which pulmonary artery pressure was monitored.

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A.1.1.17.5 Quantitative autoradiographic localization of endothelin-1 receptor

PURPOSE AND RATIONALE

The endothelin-1 (ET-1) receptor can be quantified in tissue, such as in rat hearts with chronic infarction after left coronary ligation, by computerized *in vitro* autoradiography (Kohzuki et al. 1996)

PROCEDURE

Myocardial infarction is induced in Wistar rats by left coronary artery ligation (see A.3.2.2). After various time intervals (1–8 months) the animals are decapitated, the hearts rapidly removed, and snap-frozen in isopentane at –40 °C. Frozen section (20 µm) are cut in a cryostat at –20 °C. The sections are thaw-mounted onto gelatin-coated slides, dried in a desiccator for 2 h at 4 °C and then stored at –80 °C.

Quantitative autoradiography

Radioligand: Endothelin-1 is iodinated with ^{125}Iodine using Iodogen (Pierce Chemical Co, IL, USA)

^{125}I-ET-1 binding: The sections are preincubated for 15 min at 20 °C in 20 mmol/L Hepes buffer, pH 7.4, containing 135 mmol/L NaCl, 2 mmol/L $CaCl_2$, 0.2% BSA, and 0.01% bacitracin. The sections are then incubated with 11.1 KBq/ml ^{125}I-ET-1 in the same buffer for 60 min at 20 °C. Nonspecific binding is determined in the presence of 10^{-6} mol/L ET-1. Binding isotherms are determined using a set of serial sections incubated with 10^{-12} to 10^{-6} mol/L unlabelled ET-1 for 60 min.

After incubation, the sections are rapidly dried under a stream of cold air, placed in X-ray cassettes, and exposed to Agfa Scopix CR3 X-ray film for 12–72 h at room temperature. After exposure, the sections are fixed in formaldehyde and stained with hematoxylin and eosin. The optical density of the X-ray films is quantified using an imaging device controlled by a personal computer.

EVALUATION

The optical density of the autoradiographs is calibrated in terms of the radioactivity density in dpm/mm^2 with reference standards maintained through the procedure. The apparent binding site concentration (B_{max}) and binding affinity constant (K_A) in all the areas (excluding coronary arteries) of the right ventricle, intraventricular septum, the infarcted area in the left ventricle and the non-infarcted area in the left ventricle are estimated by an iterative non-linear model-fitting computer program LIGAND (Munson and Rodbard 1980).

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A.1.1.17.6 Inhibition of endothelin converting enzyme

PURPOSE AND RATIONALE

Endothelin converting enzyme inhibitors suppress the biosynthesis of endothelin are therefore potential an-

tihypertensive drugs (De Lombaert et al. 1994; Trapani et al. 1995; Morita et al. 1994; Bihovsky et al. 1995; Claing et al. 1995; Descombes et al. 1995; Chackalamannil et al. 1996; Jeng 1997; Jeng and De Lombaert 1997; Brunner 1998).

Purification of rat and porcine endothelin converting enzyme (ECE) was reported by Ohmaka et al. (1993), Takahashi et al. 1993). Molecular cloning and characterization of the enzyme ECE-1 was performed from rat (Shimada et al. 1994), bovine (Ikura et al. 1994; Schmidt et al. 1994; Xu et al. 1994), and human tissue (Schmidt et al. 1994; Shimada et al. 1995; Yorimitsu et al. 1995).

A second enzyme, termed ECE-2, was cloned (Emoto and Yanagisawa 1995).

Walkden and Turner (1995) described the expression of endothelin converting enzyme and related membrane peptidases, e.g., the endopeptidase E-24.11, in the human endothelial cell line EA.hy926.

IN VITRO ASSAY

A rapid and selective *in vitro* assay for endothelin-converting enzyme was described by Fawzi et al. (1994). The assay is based on the quantitative determination of [^{125}I]endothelin-1 released from (3-[^{125}I]iodotyrosyl^{13})big endothelin-1 by binding to the membrane bound endothelin receptor.

PROCEDURE

For the **preparation of lung membranes**, frozen guinea pig lungs are weighed and homogenized in 10 times gram tissue weight of solution A (50 mM Tris-HCl, pH 7.4, 025 M sucrose, and 2 mM EDTA) using a Polytron tissue homogenizer. Homogenization is repeated 4 times with 5- to 8-min intervals between homogenization. Homogenates are spun for 30 min at 2000 *g*. Supernatants containing membranes are carefully decanted and saved. Pellets are rehomogenized in solution A and homogenates are spun at 2000 *g* for 30 min. Supernatants are removed, mixed with supernatants from the first spin and spun at 100 000 *g* for 60 min. Pellets containing membranes are suspended in solution B (10 mM Tris-HCl, pH 7.4, and 0.125 M sucrose) using a Dounce homogenizer. Samples are divided into 1-ml fractions, rapidly frozen in a dry ice-methanol bath, and stored at −80 °C.

Rat liver membranes are prepared with the same method and further purified over a sucrose step gradient. The membranes are suspended in solution C (10 mM Tris-HCl, pH 7.4) containing 44% sucrose at a protein concentration of 2 mg/ml. Samples of 25 ml are placed in ultraclear centrifuge tubes for the Beckman SW 28 rotor, overlaid with 10 ml solution C containing 42.3% sucrose, and spun for 2 h at 27 000 rpm (100 000 *g*). Top layers containing membrane are collected and diluted with solution C to obtain an 8% sucrose concentration. Samples are spun in a 45 Ti rotor (100 000 *g*) for 1 h. Supernatants are discarded. Pellets containing membrane are suspended in solution B, divided into 1-ml samples, rapidly frozen in dry ice-methanol bath, and stored at −80 °C.

For the **endothelin (ET) binding assay**, membrane preparations are incubated with selected concentrations of [^{125}I]endothelin-1 (final reaction volume = 500 µl) in a solution D containing 60 mM Tris-HCl, pH 7.4, 150 mM NaCl and 6 mg/ml BSA for 90 min at 37 °C. Reactions are terminated by the addition of 4 ml of solution E containing 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl at 4 °C followed by rapid filtration on Whatman GF/B glass microfiber filters. Filters are presoaked for 1 h at 4 °C in a solution containing 50 mM Tris-HCl, pH 7.4, 10 mg/ml BSA and 0.1% sodium azide. Test tubes and filters are washed four times with 4 ml of solution E at 4 °C, and radioactivity retained on the filters is counted in a gamma counter. Nonspecific binding is determined in the presence of 1 µM unlabeled ET-1 in the reaction mixture.

For the **endothelin converting enzyme assay**, samples containing 10 µg of protein are incubated in a solution containing 50 mM Tris-HCl, pH 7.0, 100 mM NaCl and 5 mg/ml BSA in a final volume of 100 µl. Conversion reactions are initiated by addition of [^{125}I]big endothelin-1 to obtain a final concentration of 500 pM. Samples are incubated for 2 h at 37 °C. To measure [^{125}I]endothelin-1 released from [^{125}I]big endothelin-1 conversion, 50 µg of purified rat liver membranes (as a source of ET receptors) is added to the reaction mixture, and reaction volume is adjusted to 500 µl and solution composition is adjusted to that of solution D of the endothelin binding assay. Following a 90-min incubation at 37 °C to reach equilibrium in binding, reactions are terminated by addition of a solution E at 4 °C followed by rapid filtration on Whatman GF/B glass microfiber filters. Nonspecific binding is determined in the presence of 1 µM unlabeled ET-1 in the reaction mixture. Specific ET-1 binding is used as an index of endothelin converting enzyme activity.

To test the effect of endothelin converting enzyme inhibitors, endothelin converting enzyme assays are carried out in the presence of desired concentrations of the compounds.

EVALUATION

Endothelin converting enzyme activity in the presence of compounds is expressed as a percentage of control endothelin converting enzyme activity in the membrane preparation which is determined simultaneously. The concentration of compounds producing a 50% inhibi-

tion of endothelin converting enzyme activity (IC_{50} values) is determined from a plot of the percentage of control endothelin converting enzyme activity versus log concentration of compounds.

IN VIVO ASSAY

PROCEDURE

Male Sprague Dawley rats weighing 300–400 g are anesthetized with ether, spinalized and placed under artificial respiration. The vagus nerves are cut and the carotid arteries ligated. A catheter is placed in one of the carotid arteries to allow measurement of arterial blood pressure. The second catheter in placed into the penile vein to allow infusion or injection of drugs. After stabilization, the animals receive a first injection of either ET-1, big ET-1, norepinephrine, angiotensin I or AT II, The pressor responses are recorded and after return to the baseline, a second injection of the agonist is given either in the presence or the absence of the inhibitor.

EVALUATION

Data are calculated as mean ±SEM. Student's *t*-test for paired and unpaired observations is used to analyze the results.

MODIFICATIONS OF THE METHOD

Little et al. (1994) developed a two-step protocol for high-throughput assays of endothelin converting enzyme activity. Human umbilical vein and human aorta endothelial cells were found to preferentially convert the big endothelin-1 isopeptide through a membrane-bound, thiorphan-insensitive, and phophoramidon-sensitive zinc metalloendopeptidase. Endothelins are quantified by a separate step using either enzyme immunoassays or radioceptor assays in 96-well formats. The method can be used to either characterize ECE from different tissues or screen for inhibitors of a specific ECE activity.

McMahon et al. (1993) tested the effects of endothelin converting enzyme inhibitors and endothelin receptor subtype A antagonists on blood pressure in spontaneously and renal hypertensive rats.

Changes of vascular resistance in isolated perfused kidneys were used by Descombes et al. (1995) to characterize a selective inhibitor of big ET-1 responses. The studies were performed on kidneys taken from adult male Wistar rats (300–400 g). The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and the left kidney was prepared for infusion with Tyrode solution. The changes in renal vascular resistance were recorded as changes in perfusion pressure monitored at constant flow (6 ml/min). After stabilization, a bolus injection of ET-1 or big ET-1 was administered and the resulting pressure responses were recorded. On return to baseline levels, a second injection of the endothelins was given either under control conditions or in presence of the putative enzyme inhibitor.

Because increasing evidence implicates that endothelin plays a role in the pathophysiology of cerebral insults, Kwan et al. (1997) studied the prevention and reversal of cerebral vasospasm in an experimental model of subarachnoid hemorrhage. Three ml of arterial blood was withdrawn from the ear artery of rabbits and injected into the cisterna magna under anesthesia. Drugs were administered either before or 24 h after this procedure. Forty-eight hours later, the animals were anesthetized again and perfusion fixation was performed with Hank's balanced salt solution followed by a mixture with 2% paraformaldehyde and 2.5% glutaraldehyde. Cross sections of the basilar arteries were analyzed by computer-assisted morphometry.

A review on the knowledge of molecular pharmacology of endothelin converting enzymes was given by Turner and Murphy (1996).

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A.1.1.18
Nitric oxide

A.1.1.18.1
General considerations on nitric oxide

PURPOSE AND RATIONALE

The endothelium releases a labile, diffusable vasorelaxing substance that has been termed endothelium-derived relaxing factor = EDRF. (Furchgott and Zawadszki 1980). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor (Palmer et al. 1987).

Nitric oxide plays a role in a wide range of physiological processes including regulation of blood flow and arterial pressure via endothelium-dependent relaxation of blood vessels (Rees et al. 1989; Moncada et al. 1991; Umans and Levi 1995), peripheral nitregric transmission at smooth muscle (Rand and Li 1995), intracellular communication in the CNS with activation of guanylyl cyclase in target neurons (Southam and Garthwaite 1993), in neurogenic inflammation (Kajekar et al. 1995), and macrophage defense mechanisms following exposure to bacterial products (Förstermann et al. 1992; Förstermann and Kleinert 1995; Knowles and Moncada 1994). NO-donor drugs, such as sodium nitrite, sodium nitroprusside, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), 3-morpholinosydnonimine (SIN-1) are used as vasodilators (Schör et al. 1989). N^G-Nitro-L-arginine was described as an antagonist of endothelium-dependent dilator responses by inhibiting endothelium-derived relaxing factor release (Moore et al. 1990; Lamontagne et al. 1991). Ribero et al. (1992) proposed inhibition of nitric oxide synthesis by long-term treatment of rats with nitro-L-arginine as a new model of arterial hypertension.

Excessive production of NO damages DNA and activates poly(ADP-ribose)polymerase (PARP) (Pieper et al. 1999). In cases of massive NO production, neurons enter the PARP-suicide pathway. NO damages DNA via to major pathways: the first involves nitrosation of primary or secondary amines and nucleic acid bases, whereas the second involves the combination of NO with superoxide to from peroxinitrite (Szabó et al. 1996, 1997). The most likely reactive oxidant intermediate responsible for DNA breakage is peroxynitrous acid which rapidly oxidizes sulphydryl groups, and also nitrates and hydroxylates aromatic compounds including tyrosine, tryptophan, and guanosine (Halliwell 1997). Downstream DNA damage that follows excessive NO production results in significant activation of poly(ADP-ribose)polymerase which leads to rapid energy depletion and cell death.

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A.1.1.18.2 Bioassay of EDRF release

PURPOSE AND RATIONALE

EDRF release from arterial endothelium can be studied by a sandwich technique using donor tissue with intact endothelium facing with its intimal side the intimal side of a detector tissue.

PROCEDURE

Rabbits are subjected to various kinds of treatment, e.g., atherogenic diet or drug treatment for prevention of arteriosclerosis. Aorta segments, about 2 cm in length, are prepared, cut open along their longitudinal axis and pinned to a tissue suspender without damaging the endothelium. These segments serve as donor tissue for EDRF. Circumferential aorta strips from the abdominal aorta of untreated control rabbits are de-endothelialized by gently blotting their luminal surfaces on wet filter paper. These denuded abdominal aorta strips are pinned opposite the donor segments (intimal surface facing intimal surface) and function as detector for luminally released EDRF. Each sandwich preparation is suspended in a 40-ml organ bath, filled with oxygenated Krebs-Ringer buffer at 37 °C containing 10 mM indomethacin. After connecting the detector strip to a force transducer, the angle between the detector strip and the donor segment is minimized and the distance between donor and detector tissue standardized. After one hour stabilization, the strips are brought to their optimum length-tension relationship by repeated exposure to 80 mM KCl. When a stable contractile response is established, the strips are precontracted with phenylephrine to 80–100% of their KCl-induced contraction. After stabilization of plateau phase, cumulative doses of acetylcholine (0.01–10 mM) are added to induce EDRF release from donor tissues.

EVALUATION

Relaxations of the detector strip induced by EDRF release from treated donor rabbit aortas are compared with aortas from control rabbits.

MODIFICATIONS OF THE METHOD

An other bioassay for measuring function of cultured endothelial cells using a computer system for the acquisition and analysis of vascular contractility has been published by Winn et al. (1992).

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A.1.1.18.3 Isolated arteries with and without endothelium

PURPOSE AND RATIONALE

Endothelial cells are able to synthesize and release potent vasoconstrictive agents, such as endothelin and angiotensin as well as vasodilating agents, such as EDRF. In isolated arterial segments the endothelial surface can be functionally destroyed allowing a differentiation between a direct action of drugs on the smooth muscle cells and an indirect effect via the endothelium. Isolated rings of rabbit or rat aorta are useful models to study the effects of endothelium derived factors such as EDRF or endothelins and their antagonists (Linz et al. 1986; Tracey et al. 1990; Fujimoto et al. 1992; Fukuroda et al. 1992; Wiemer et al. 1992). A survey of the history and on techniques leading to the discovery of endothelium-dependent relaxation was given by Furchgott (1993).

PROCEDURE

The descending thoracic aorta from rabbits of either sex (weighing 2.5–3.5 kg) is excised and dissected free from connective tissue. Care is taken to avoid damage of the endothelium. The aorta is divided into 2 mm wide rings and cut off in small strips. From some strips, the endothelium is removed by gently rubbing the intimal surfaces between the fingers for approximately 30 s. The strips are suspended in a 25 ml organ bath containing Krebs-bicarbonate solution at 37 °C being gassed continuously with 5% CO_2 / 95% O_2. Contractions of the strips are recorded isometrically with a load of 2 g on the tissues. After an equilibration period of 2 h a stable baseline tone is reached.

To study the vasodilating effects of a compound, the strips are contracted with norepinephrine (10^{-8} M), or angiotensin II (10^{-7} M), or potassium chloride (20 mM). When a stable contraction plateau has been reached, the vasodilating agent is added in various concentrations. In these concentrations, norepinephrine, angiotensin II and KCl evoke a response of 60–80% of maximal contraction in intact rings of rabbit aorta. Rings without endothelium exhibit a response which is significantly enhanced in comparison with the response of the intact preparation after norepinephrine and angiotensin II precontraction.

To indicate the functional removal of the endothelium, the responsiveness of each preparation is tested with the known endothelium-dependent dilator, acetylcholine. In endothelium-intact rings, acetylcholine relaxes contractions induced by norepinephrine or angiotensin II. In precontracted rings devoid of endothelium, acetylcholine does not show any relaxing effect or causes contractions by itself at higher concentrations (Furchgott and Zawadzki 1980). As an example, atriopeptin III causes a similar concentration-dependent relaxation of all precontracted preparations with intact and with functionally destroyed endothelium indicating a direct effect on the smooth muscle cells. The relaxation is accompanied by an increase of cGMP.

EVALUATION

Statistical analyses are performed by regression analysis of dose response curves to determine EC_{50} values. Data are given as means ± standard deviation.

CRITICAL ASSESSMENT OF THE METHOD

The isolated aortic ring of rabbits with and without functionally intact endothelium is a useful tool to differentiate direct effects on the arterial smooth musculature from effects mediated by the endothelium.

MODIFICATIONS OF THE METHOD

Fujimoto et al. (1992) used the thoracic aorta from rats to study the effects between endothelin and an endothelin receptor antagonist. In transverse strips from *rat* thoracic aorta, 2 mm wide and 4–5 mm long, the endothelium was removed by gently rubbing the interior surface of the aorta. Concentration-response curves of contractions after ET-1 in the presence and the absence of the inhibitor were compared.

Pellisier et al. (1992) perfused the isolated mesenteric vascular bed of the **rat** with Tyrode solution and measured the perfusion pressure after injection of graded doses of norepinephrine and the dose-dependent relaxation due to acetylcholine in the vascular bed precontracted by norepinephrine infusion. In order to destroy the endothelial layer, the perfusate was changed to a hypotonic Tyrode solution containing all of the constituents present in normal Tyrode solution but in one-tenth of the concentration resulting in disruption of more than 95% of the endothelial cells. The effect of norepinephrine was enhanced, whereas the effect of acetylcholine was abolished.

Legan and Sisson (1990) described a method to denude **rat** aortic endothelium *in vitro* with saponin.

Bohn and Schönafinger (1989) used helical strips of pulmonary arteries of **guinea pigs** in which the endothelium has been removed for biological detection of NO.

Fukuroda et al. (1992) used spiral strips from **porcine** coronary artery and vein and from intrapulmonary artery and vein removing the intimal surface by lightly rubbing with wet filter paper. Concentration-

contraction curves for ET-1 and ET-3 were obtained with and without an endothelin antagonist.

Hayashi et al. (1988) described functional and anatomical recovery of endothelium after balloon denudation of the left circumflex coronary artery in **dogs**.

Endothelial denudation of the left circumflex coronary artery was used by Chu and Cobb (1987) to study the vasoactive effects of serotonin on proximal coronary arteries in awake **dogs**.

Experiments in isolated rings of the left circumflex or left anterior descending coronary artery of **dogs** with and without endothelium were performed by Desta et al. (1995).

Terrón (1996) analyzed the effects of 5-HT_1-receptor antagonists on 5-HT and sumatriptan induced isometric contractions in endothelium denuded segments of **canine** coronary arteries.

Ren et al. (1993) isolated coronary arteries from Japanese **monkeys** (*Macaca fuscata*) with and without endothelium to study muscarinic receptor subtypes mediating vasodilatation and vasoconstriction.

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A.1.1.18.4 Nitric oxide formation by cultured endothelial cells

PURPOSE AND RATIONALE

Endothelial cells are able to synthesize and to release not only potent vasoconstrictor peptides such as angiotensin and endothelin but also potent dilators such as nitric oxide (NO), ATP, substance P, and bradykinin.

NO-formation can be assessed by determination of intracellular cyclic GMP in cultured endothelial cells, whereas release of NO from these cells can be measured by the stimulatory effect of NO on the activity of soluble guanylyl cyclase (Lückhoff et al. 1988; Wiemer et al. 1991; Linz et al. 1992; Bogle et al. 1992; review by Moncada et al. 1991).

PROCEDURE

Endothelial cell culture

Bovine or porcine aorta is obtained from local slaughter houses. Endothelial cells are isolated by digestion with dispase (Lückhoff et al. 1988). The cells are seeded on 6- or 24-well plates (e.g., Nunc Intermed®,

Wiesbaden, Germany) and grown to confluence. Dulbecco's modified Eagle's/Ham's F-12 medium containing 20% fetal calf serum is supplemented with penicillin (10 U/ml), streptomycin (10 µg/ml), L-glutamate (1 mM/l), glutathione (5 mg/ml), and L(+)ascorbic acid (5 mg/ml); (Biotect® protection medium).

Measurement of cyclic GMP

Primary cultures of endothelial cells are used. After removal of the culture medium by aspiration, the monolayer is washed twice with 2 ml HEPES-Tyrode's solution (37 °C). Thereafter, the cells are preincubated for 15 min at 37 °C with 3-isobutyl-1-methyl-xanthine (IBMX), (10^{-4} M/l). After this time, drugs or solvents are added. After predetermined periods, the incubation medium is quickly removed. The cells are then immediately extracted with 0.6 ml 6% trichloro-acetic acid and scraped off with a rubber scraper. The cell suspension is sonicated for 10 s before being centrifuged for 5 min at 4 000 *g*. The supernatants are extracted with four volumes of water saturated diethylether, and the samples frozen (–20 °C) until analysis. The protein contents of the samples are measured according to Lowry et al. (1951). Cyclic GMP can be determined in the acetylated samples by various methods (Heath et al. 1992), e.g., using a commercially available radio-immunoassay (New England Nuclear). Cyclic GMP content is expressed as picomoles GMP per milligram protein.

Measurement of NO release

Release of NO from endothelial cells is assayed on the basis of the stimulatory effect of NO on the activity of soluble guanylyl cyclase (purified from bovine lung) (Gerzer et al. 1981). The activity of the enzyme is determined in terms of the formation of cyclic [^{32}P]GMP from α-[^{32}P]GTP. Reactions are carried out in a reaction mixture containing 30 mM triethanolamine-HCl (pH 7.4), 1 mM reduced glutathione, 4 mM $MgCl_2$, 1 mM cGMP and 0.1 mg/ml bovine γ-globulin (total volume of 0.18 ml) at 37 °C in the presence of α-[^{32}P]GTP (0.03 mM; 0.2 µCi) and soluble gua-nylyl cyclase (4 µg). Ten-µl samples are quickly transferred to the reaction mixture. Enzymatic formation of cGMP is allowed to proceed for 60 s and then stopped by the addition of 450 µl zinc acetate (120 mM) and 500 µl sodium carbonate (120 mM). A complete inhibition of cGMP formation can be achieved by pre-incubation of the monolayers for 30 min with the stereospecific inhibitor of NO synthase, N^G-nitro-L-arginine.

EVALUATION

Time-response curves and dose-response curves after addition of various activators or inhibitors of NO synthase are established. Data are reported as mean values ±SEM of cGMP (pmol/mg protein) or guanylyl cyclase activity (nmol/mg/min). Statistical evaluation is performed with Student's *t*-test.

MODIFICATIONS OF THE METHOD

Isolation of porcine cerebral capillary endothelial cells has been described by Wiemer et al. (1994).

Feelisch and Noack (1987) and Nakazawa et al. (1992) used chemiluminescence techniques for determination of NO.

A method for on-line detection of nitric oxide formation in liquid aqueous phase by electron paramagnetic resonance spectroscopy was described by Mordvintcev et al. (1991). Similar methods were used by Ichimori et al. (1992), Lancester et al. (1992), Steel-Goodwin et al. (1992).

Hecker et al. (1995) used a cascade superfusion bioassay to characterize a stable L-arginine-derived relaxing factor released from cytokine-stimulated vascular smooth muscle cells.

Electrochemical microprobes for direct measurement of NO in tissues have been developed (Shibuki 1990; Ishida et al. 1996; Smits and Lefebvre 1997).

Malinski and Taha (1992), Linz et al. (1999) measured nitric oxide release by a porphyrinic-based microsensor with a detection limit of 10 nmol/L. The amperometric signal at a constant potential of 0.67 V was measured with a voltametric analyzer (PAR model 273, Princeton Applied Research) interfaced with an IBM 80486 computer with data acquisition and software.

Gabriel et al. (1997) developed a method for the detection of intracellular nitric oxide generation in dissociated cerebellar granule cells using dichlorofluorescin diacetate and flow cytometry.

Sumpio et al. (1987) found that cyclic mechanical stress stimulates cultured bovine aortic endothelial cells to proliferate.

Using this method, Rosales et al. (1997) found that exposure of endothelial cells to cyclic strain induces elevations of cytosolic Ca^{2+} concentration through mobilization of intracellular and extracellular pools.

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A.1.1.18.5 Expression of nitric oxide synthase

PURPOSE AND RATIONALE

Properties of various forms of nitric oxide synthase (NOS) have been described by Mayer et al. (1992), Leone et al. (1992), Hevel et al. (1992), Förstermann et al. (1992, 1995), (Salter et al. 1992), Pollock et al. (1992), Schmidt et al. (1992), among them type I, which is constitutively expressed in neurons; the inducible type II which is found in macrophages and hepatocytes, but also in the brain (Moro et al. 1998), where it may contribute to NO-mediated neurotoxicity; and type III, which is constitutively expressed in endothelial cells (Knowles and Moncada 1994). NOS can be inhibited by several routes, e.g., competition with L-arginine, NADPH, flavin or tetrahydrobiopterin, interaction of the haeme group of NOS, interference with Ca^{2+} availability or calmodulin binding to the enzyme (Fukuto and Chaudhuri 1995). A widely used inhibitor is L-NAME (Vargas et al. 1991). Selective inhibition of constitutive NOS can by achieved by 7-nitroindazole (Moore et al. 1993); of the inducible NOS by aminoguanidine hydrochloride (Griffiths et al. 1993) and by 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (= AMT) (Nakane et al. 1995). Linz et al. (1999) determined NOS in the left cardiac ventricle of hypertensive rats.

PROCEDURE

Tissues are ground at the temperature of liquid nitrogen using a microdismembranator (Braun). The powders are extracted for 1 h on ice with 10 mmol/l Tris-HCl, pH 7.4, containing 1% SDS and protease inhibitors (complete, Boehringer Mannheim). Debris is removed by a 30-min centrifugation at 4 °C (>100 000 *g*). 100 µg of total of the protein extracts are subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Hybond, Amersham). The eNOS protein is detected by use of a specific antibody (monoclonal anti-NOS III), Transduction Laboratories) and visualized by enhanced chemifluorescence with a commercially available kit (Amersham). As a secondary antibody, an anti-mouse IgG antibody coupled to alkaline phosphatase is used (Jackson ImmunoResearch Laboratories). Chemifluorescence is analyzed and quantified by scanning with a Fluorimager 595-system (Molecular Dynamics).

EVALUATION

The data are given as mean ±SEM. ANOVA is used followed by Tuckey's test for post-ANOVA multiple pair comparisons.

MODIFICATIONS OF THE METHOD

Linz et al. (1997) measured expression of ecNOS in the carotid artery of hypertensive rats by Western blot analysis. Frozen (–70 °C) vessels were thawed and extracted with guanidium isothiocyanate/phenol/chloroform (Chomczynski and Sacchi 1987). Crude protein fractions were obtained by alcohol precipitation of the phenol phase. A total of 100 µg of the protein extracts was subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Ponceau staining was performed to verify the quality of the transfer and the equipartition of protein in each lane. EcNOS protein was detected with a specific antibody (mouse NOS III, Tansduction Laboratories) and visualized by enhanced chemifluorescence with a commercially available kit (Amersham). The autoradiographs were analyzed by scanning densitometry.

McCall et al. (1991) identified N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase.

Bauersachs et al. (1998, 1999) measured vascular reactivity in isolated rat aortic rings mounted in an organ bath (Föhr Medical Instruments, Seeheim Germany) for isometric force measurement and determined superoxide anion production by lucigenin-enhanced chemiluminescence and endothelial nitric oxide synthase and soluble guanylyl cyclase expression by reverse transcription-polymerase chain reaction.

Von der Leyen et al. (1995) reported gene therapy inhibiting neointimal vascular lesions in rats. After denudation of the endothelium of carotid arteries by balloon injury, endothelial cell nitric oxidase expression in the vessel wall was restored by using the Sendai virus/liposome *in vivo* gene transfer technique.

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A.1.1.19 Inhibition of Na^+/H^+ exchange

PURPOSE AND RATIONALE

Na^+/H^+ exchange was first described by Murer et al. (1976) in a study of intestinal and renal brush border vesicles. The plasma membrane Na^+/H^+ exchanger is an ubiquitous pH regulating cellular ion transport system. It is driven by the Na^+ gradient and extrudes protons from the cytosol in exchange for extracellular Na^+ ions (Aronson 1985; Frelin et al. 1988; Fliegel and Dyck 1995; Orlowski and Grinstein 1997; Wakabayashi et al. 1997; Dibrov and Fliegel 1998). Six mammalian Na^+/H^+ exchangers: NHE1, NHE2, NHE3; NHE4, NHE5 (Attaphitaya et al. 1999; Szabo et al. 2000), and NHE6 have been described (Tse et al. 1994; Orlowski 1999; Counillon and Pouysségur 2000).

In cardiac tissue the exchanger has a major role in the control of intracellular pH. At the onset of cardiac ischemia and during reperfusion, Na^+/H^+ exchange is excessively activated by low intracellular pH. Since the deleterious Na^+ influx in this condition was found to originate mainly from Na^+/H^+ exchange (Frelin et al. 1984; Schömig et al. 1988), the exchanger seems to be responsible for an increase of cytosolic sodium in ischemic cells. The accumulation of intracellular Na^+ causes an activation of Na^+/K^+ ATPase (Frelin et al. 1984; Rasmussen et al. 1989) which in turn increases ATP consumption.

During ischemia the aerobic metabolism of glucose terminates in lactic acid. A vicious circle leads to a further decrease of intracellular pH and to a further activation of Na^+/H^+ exchange, resulting in energy depletion, cellular Na^+ overload and finally due to the coupling of Na^+ and Ca^{2+} transport via Na^+/Ca^{2+} exchange, cellular Ca^{2+} overload (Lazdunski et al. 1985;

Tani and Neely 1990; Scholz and Albus 1993). Especially in ischemic cardiac tissue, where Na^+/H^+ exchange is the predominant pH regulating ion transport system (Weissenberg et al. 1989), these pathological events can lead to increased excitability and precipitation of cellular death. Therefore, it is desirable to find potent and well tolerated inhibitors of Na^+/H^+ exchange which should be able to interrupt this vicious cycle, to conserve cellular energy stores and to diminish cellular excitability and necrosis during cardiac ischemia. Such effects have been found with relatively weak inhibitors of Na^+/H^+ exchange at high toxic doses, such as amiloride and ethyl isopropyl amiloride (Scholz et al. 1992).

More potent Na^+/H^+ exchange inhibitors showed beneficial effects on ischemia/reperfusion injury (see A.5.0.7 and A.5.0.8) in rats (Aye et al. 1997; Myers et al. 1998; Aihara et al. 2000), dogs (Gumina et al. 1998, 2000) and pigs (Portman et al. 2001). Heart hypertrophy and heart failure after myocardial infarction is reduced (Yoshida and Karmazyn 2000; Kusumoto et al. 2001). Ischemia-induced apoptosis in isolated rat hearts is attenuated by sodium-hydrogen exchange inhibitors (Chakrabarti et al. 1997).

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A.1.1.19.1 Inhibition of Na^+/H^+ exchange in thrombocytes

PURPOSE AND RATIONALE

The inhibition of Na^+/H^+ exchange has been studied in platelets by measuring the optical density after osmotic cell swelling (Rosskopf et al. 1991).

PROCEDURE

About 5 ml blood is withdrawn by venipuncture from human donors or from the vena jugularis externa of Beagle dogs or from the aorta of anesthetized Wistar rats (weighing 250–350 g). Coagulation is inhibited by 0.8 ml citrate acid dextrose (65 mM citric acid, 11 mM glucose, 85 mM trisodium citrate). Platelet-rich plasma (PRP) is obtained by centrifugation of whole blood at 90 *g* for 10 min at room temperature. Platelet count is measured, e.g., with a Casey 1 multichannelyser (Schärfe System, Reutlingen, Germany)

Each of the experiments is performed with 10–50 µl PRP containing 20×10^6 platelets in a volume of 100 µl with saline. To activate Na^+/H^+ exchange in the platelets by intracellular acidification, 500 µl propionate buffer (135 mM Na-propionate, 1 mM HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 20 mM HEPES, pH 6.7, 22 °C) are added to the PRP/NaCl solution. Swelling of the platelets results in a decrease of optical density which can be measured with an aggregometer, e.g. with a Turbitimer (Behringwerke, Marburg, Germany). The system is activated photometrically by the addition of the propionate buffer to the cuvette. The experiments are performed with and without the addition of the Na^+/H^+ exchange inhibitor to be tested. The inhibitors are added in concentrations between 10^{-4} and 10^{-8} mol/l. 5-(N-ethyl-N-isopropyl)amiloride (EIPA) is used as standard. During the experiments all solutions are kept at 22 °C in a temperature controlled water bath.

EVALUATION

Results are given as means ±SD. Student's *t*-test is employed for statistical evaluation. IC_{50} values are calculated from dose-response curves.

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A.1.1.19.2 Inhibition of Na^+/H^+ exchange in cholesterol activated rabbit erythrocytes

PURPOSE AND RATIONALE

The inhibition of Na^+/H^+ exchange has been studied in cholesterol activated rabbit erythrocytes by flame photometry of sodium (Scholz et al. 1992, 1993).

PROCEDURE

White rabbits (New Zealand strain, Ivanovas) are fed with a rabbit standard chow with 2% cholesterol for 6 weeks to increase the Na^+/H^+ exchange (Scholz et al. 1990) and to make the erythrocytes suitable for measurement of sodium influx via Na^+/H^+ exchange by flame photometry. Blood is drawn from the ear artery of the rabbits and coagulation prevented with 25 IU/ml potassium heparin. The hematocrit of the samples is determined in duplicate by centrifugation. Aliquots of 100 µl are taken to measure the initial sodium content of the erythrocytes.

To determine the amiloride sensitive sodium influx into erythrocytes, 100 µl of each blood sample are added to 5 ml of buffer made hyperosmolar by sucrose (140 mM NaCl, 3 mM KCl, 150 mM sucrose, 0.1 mM ouabain, 20 mM tris-hydroxy-methylamino-methane, pH 7.4) and incubated for 60 min at 37 °C. Subsequently, the erythrocytes are washed three times in ice-cold $MgCl_2$-ouabain-solution (112 mM $MgCl_2$, 0.1 mM ouabain).

For determination of intracellular sodium content, the cells are hemolyzed in distilled water, the cell membranes are centrifuged and the sodium concentration of the haemolysate is measured by flame photometry. Net influx of sodium into the erythrocytes is calculated from the difference between the initial sodium content and the sodium content after incubation. Amiloride-sensitive sodium influx is calculated from the difference between sodium content of erythrocytes incubated with and without amiloride (3×10^{-4} M). Each experiment is done with the erythrocytes from 6 different animals. In each case, the comparison of Na^+ contents is based on erythrocytes from the same animal. Doses between 10^{-4} and 10^{-7} M of the inhibitor are tested.

EVALUATION
Statistical analysis of the data obtained is performed with Student's *t*-test for paired groups. IC_{50} values are calculated from dose-response curves.

REFERENCES
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Scholz W, Albus U, Lang HJ, Linz W, Martorana PA, Englert HC, Schölkens BA (1993) Hoe 694, a new Na^+/H^+ exchange inhibitor and its effects in cardiac ischemia. Br J Pharmacol 109:562–568

A.1.1.19.3 Sodium influx into cultured cardiac myocytes

PURPOSE AND RATIONALE
The inhibition of Na^+/H^+ exchange has been studied in cultured cardiac myocytes (Scholz et al. 1992).

PROCEDURE
Rat myocardial cells are isolated from hearts of neonatal rats by trypsin digestion. The cells are cultured in 35 mm dishes and grown to confluence in Dulbecco's Minimum Essential Medium (DMEM, GIBCO®) in an atmosphere containing 10% CO_2. After confluence, the cells are used for measurement of $^{22}Na^+$ influx. The cells are washed twice with Krebs-Ringer-solution buffered with HEPES/Tris (KRB) in which sodium chloride has been replaced by choline chloride (Choline chloride 130 mM, $CaCl_2$ 1.5 mM, KCl 5 mM, $MgCl_2$ 1 mM, HEPES 20 mM, pH 7.0 with Tris) and then incubated for 20 min at 37 °C in the same buffer with added 0.1% bovine serum albumin (BSA) and 10 mM/l glucose. The culture dishes are then incubated for another 10 min with Na^+-propionate for cytosolic acidification and stimulation of Na^+/H^+ exchange. The compounds are dissolved in 500 µl/dish KRB in which 50% of the sodium chloride has been replaced by choline chloride containing additionally 2 µCi/ml $^{22}Na^+$-bicarbonate, and 5-(N-ethyl-N-isopropyl)amiloride (EIPA). After the stimulation period, sodium influx is terminated by washing the cells twice with ice-cold stop solution (0.1 mM $MgCl_2$, 10 mM Tris, pH 7.0). Subsequently, the cells are lyzed with 250 µl trichloro-acetic acid and scraped from the dishes. Radioactivity is determined in a Packard gamma counter. Doses between 3×10^{-4} and 10^{-8} mM/l of standard and new compounds are tested. Six dishes are used for each concentration of test compounds.

EVALUATION
Mean values ±SD are compared with Student's *t*-test. IC_{50} values are calculated from dose-response curves.

REFERENCES
Scholz W, Albus U, Linz W, Martorana P, Lang HJ, Schölkens BA (1992) Effects of Na^+/H^+ exchange inhibitors in cardiac ischaemia. J Mol Cell Cardiol 24:731–740

A.1.1.19.4 Inhibition of Na^+/H^+ exchange into cultured aortic endothelial cells

PURPOSE AND RATIONALE
The inhibition of Na^+/H^+ exchange has been studied in endothelial cells (Scholz et al. 1993) by measuring the $^{22}Na^+$ influx.

PROCEDURE
Bovine aortic endothelial cells (BAEC) are isolated by dispase digestion form bovine aorta obtained from animals killed at the local slaughter house. The cells are cultured in 35 mm dishes and grown to confluence in Dulbecco's Minimum Essential Medium (DMEM, GIBCO®) in an atmosphere with 10% CO_2. Three days after confluence the cells are used for measurement of $^{22}Na^+$ influx. The cells are washed twice with Krebs-Ringer-solution buffered with HEPES/Tris (KRB) in which sodium chloride has been replaced by choline chloride (Choline chloride 130 mM, $CaCl_2$ 1.5 mM, KCl 5 mM, $MgCl_2$ 1 mM, HEPES 20 mM, pH 7.0 with Tris) and then incubated for 20 min at 37 °C in the same buffer with added 0.1% bovine serum albumin (BSA) and 10 mM glucose. To stimulate Na^+/H^+ exchange the culture dishes are incubated for another 10 min with 500 µl/dish KBR in which all sodium chloride has been replaced by 65 mM each of choline chloride and Na^+-propionate or with KBR in which 50% of the sodium chloride has been replaced by choline chloride for unstimulated controls. In addition, the buffer contains 2 µCi/ml $^{22}Na^+$ and the test compounds or the standard. After the stimulation period, the sodium influx is terminated by washing the cells twice with ice-cold stop solution (0.1 mM $MgCl_2$, 10 mM Tris, pH 7.0). Subsequently, the cells are lyzed with 250 µl trichloro-acetic acid and scraped from the dishes. Radioactivity is determined in a

Packard gamma counter. Doses between 10^{-5} and 10^{-7} mM/l of standard and new compounds are tested. Six dishes are used for each concentration of test compounds.

EVALUATION

Mean values ±SD are compared with Student's *t*-test. IC_{50} values are calculated from dose-response curves.

MODIFICATIONS OF THE METHOD

Ewart et al. (1997) studied lipoprotein lipase activity in cultured rat cardiomyocytes in the presence of insulin and dexamethasone.

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Scholz W, Albus U (1993) Na^+/H^+ exchange and its inhibition in cardiac ischemia and reperfusion. Bas Res Cardiol 88: 443–455

A.1.1.19.5 NHE activity measured by intracellular pH in isolated ventricular myocytes

PURPOSE AND RATIONALE

Changes of the intracellular pH of cultured bovine endothelial cells have been fluorometrically monitored using the pH-dye 2',7'-bis(carboxyethyl)carboxyfluorescein (BCECF) by Kitazano et al. (1988). This method has been used to study the activity of inhibitors of Na^+/H^+ exchange (Scholz et al. 1995).

PROCEDURE

For preparation of isolated rat ventricular muscular cells (Yazawa et al. 1990), hearts of male Wistar rats are dissected, mounted on a Langendorff apparatus and perfused first at 37 °C for 3 min with Tyrode solution adjusted to pH 7.4, second for 5–7 min with nominally calcium free Tyrode solution and finally with calcium free Tyrode solution containing 0.12–0.2 mg/ml collagenase (Sigma type I). After 15–20 min collagenase treatment, the heart is washed with storage solution (composition in mmol/L: KOH 70, l-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 20, HEPES 10, and EGTA 0.5, pH 7.4). The ventricles are cut into small pieces and myocytes are dispersed by gently shaking and finally by filtration through a nylon mesh (365 µm). Thereafter, the cells are washed twice by centrifugation at 600–1 000 rpm for 5 min and kept at 4 °C until use. For the pH recovery experiment the cells are loaded with the membrane permeable acetoxymethyl ester (AM) form of the fluorescent indicator 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). BCECF-AM is dissolved in DMSO and diluted to a 1.25 µM storage solution. Cardiomyocytes are loaded in this solution for 30 min at room temperature and are then centrifuged and resuspended in storage solution. The measurements are performed in bicarbonate-free NaCl solution (NaCl 140, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1, glucose 10, and HEPES 10 mM/L, pH 7.4) at 34 °C using an apparatus according to Nitschke et al. (1991). The pH-dependent signal of BCECF is obtained by illuminating at 490 and 437 nm and dividing the emitted light signals (520–560 nm). The background signal, determined by closing the shutter, is subtracted from the total signal. The autofluorescence determined by illuminating unloaded cells can be ignored. In order to investigate the function of the Na^+/H^+ exchange system, the intracellular pH (pH_i) of the cells is decreased by the NH_4Cl prepulse technique and the rate of return to resting pH_i is determined. Test compounds are dissolved in the incubation medium. For each test concentration, the recovery of pH_i is first recorded in control NaCl solution.

EVALUATION

Data are analyzed by fitting a straight line to the initial (5 min) data points of the pH recovery curve. For statistical presentation, the slopes of the linear curves are demonstrated. All reported data are presented as means ±SEM. Statistical comparisons are made using either a paired or unpaired *t*-test.

MODIFICATIONS OF THE METHOD

The pH-sensitive fluorescence dye C-SNARF-1 (= carboxy-semi-naphtho-rhoda-fluor 1) was used by Yatsutake et al. (1996), Shipolini et al. (1997), and Yokoyama et al. (1998).

Fischer et al. (1999) tested new drugs for the Na^+/H^+ exchanger in Chinese hamster ovary cells which are enriched with the NHE-1 isoform of the Na^+/H^+ anti-porter. The Na^+/H^+ exchanger was stimulated with NaCl and the rate of extracellular acidification was quantified with the Cytosensor.

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A.1.1.19.6 NHE subtype specificity

PURPOSE AND RATIONALE

Molecular identification of mammalian Na^+/H^+ exchanger subtypes has been pioneered by Pouysségour and coworkers (Sardet et al. 1989) who used genetic complementation of fibroblast cell lines that lack all endogenous NHEs. Schwark et al. (1998) studied an inhibitor of Na^+/H^+ exchanger subtype 3 in various cell types.

PROCEDURE

cDNAs for the NHE subtypes human NHE1, rabbit NHE2, rat NHE3 (Pouysségour) or cloned by reverse transcriptase-polymerase chain reaction from human kidney mRNA are used. These cDNAs are cloned into the mammalian expression vector pMAMneo and transferred into the NHE-deficient mouse fibroblast cell line LAP1. Cells expressing the NHE subtypes are selected by the acid load survival method (Sardet et al. 1989). Clonal cell lines for each subtype are used for intracellular pH (pH_i) recovery after acid load. For studies of pH_i recovery (Faber et al. 1996), cells are scraped off the culture dishes washed and incubated with 5 µmol/l BCECF-AM [2',7'-bis(2-carboxyethyl)-5,6-carboxy-fluorescein-acetoxy-methyl ester] for 20 min at 37 °C in a buffer containing 20 mM NH_4Cl. The cells are then washed to remove extracellular dye and resuspended in the loading buffer without BCECF-AM. Intracellular acidification is induced by addition of 975 µl NH_4Cl-free and HCO_3^--free solution (so-called recovery medium: HCO_3^--free to inhibit the Na^+-dependent Cl^-/HCO_3^- exchanger of LAP1 cells) to an 25-µl aliquot of cells (≅25 000 cells). The pH_i recovery is recorded with a dual-grating Deltascan single-photon counting fluorometer (Photon Technology International, South Brunswick, NJ, USA) with excitation wavelength of 505 nm and 440 nm and an emission wavelength of 535 nm. The measurement time varies between subtypes (120 s for NHE1, 300 s for NHE2, 180 s for NHE3). The inhibitors are first dissolved in DMSO, diluted in recovery medium and added in a volume of 975 µl to this medium.

A cloned opossum kidney cell line (Helmle-Kolb et al. 1990) is used additionally. Cells are grown as a monolayer in growth medium (1:1 mixture of nutrient mixture Ham F12 and Dulbecco's modified medium Eagle with 10% fetal calf serum). For subcultivation and pH-recovery experiments, the cells are detached from the surface of the culture vessels with trypsin-EDTA solution (2.5 g trypsin + 0.2 g EDTA per liter in Dulbecco's phosphate-buffered saline) and suspended in growth medium. Measurement time in pH_i recovery experiments is 400 s.

Porcine renal brush-border membrane vesicles (BBMV) prepared by a Mg^{2+} precipitation technique are loaded with 150 mmol/l NaCl, 5 mmol/l HEPES/Tris, pH 7.0, and pre-incubated for 10 min at 37 °C with various concentrations of NHE inhibitors. Intravesicular acidification through Na^+/H^+ exchange is started by diluting BBMV into Na^+-free buffer (150 mmol/l tetramethylammonium chloride, 5 mmol HEPES/Tris, pH 7.0) containing the appropriate concentrations of the NHE inhibitors and the fluorescent ΔpH indicator acridine orange (12 µmol/l). The fluorescence changes of acridine orange are recorded continuously by a Hitachi F-2000 spectrofluorometer at 495 nm excitation and 525 nm emission wavelength. The initial acridine orange fluorescence quenching in controls (no inhibitor) is set to 100%.

EVALUATION

Values are presented as means ±SD (four measurement per concentration). The IC_{50} values and Hill coefficients are calculated using the Sigma plot software. Statistical significance is calculated by means of the

distribution-independent *H*-test and non-parametric *U*-test. $P < 0.05$ is considered as significant.

MODIFICATIONS OF THE METHOD

Counillon et al. (1993), Scholz et al. (1995) determined the NHE subtype specificity of Na^+/H^+ antiporters by their ability to inhibit initial rates of amiloride sensitive $^{22}Na^+$ uptake in fibroblast cell lines separately expressing the NHE-1, NHE-2 and NHE-3 isoforms.

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A.1.1.20 Inhibition of phosphodiesterase

PURPOSE AND RATIONALE

The enzyme phosphodiesterase (PDE) exists in various forms. At least 11 families of phosphodiesterases have been identified (Torphy and Page 2000).

The inhibition of cAMP-PDE and cGMP-PDE by various test compounds can be measured using a two-step radioisotopic procedure.

PROCEDURE

Materials

[8-^{3}H]cAMP (28 Ci/mmol), [8-^{3}H]-cGMP (15 Ci/mmol) and [U^{14}C]guanosine (528 mCi/mmol) are obtained from Du Pont de Nemours (Paris, France). Unlabelled cyclic nucleotides, 5'-nucleotidase (Ophiophagus hannah venom) are from the Sigma Chemical Co. (La Verpillére, France).

Tissue preparations

Male Sprague-Dawley rats (250–300 *g*) are decapitated. Hearts are perfused with 0.15 M NaCl through the aorta to remove the blood. The ventricles are minced in 5 vol. of 10 mM Tris-HCl buffer containing 0.32 M sucrose, 1 mM EDTA, 5 nM DTT and 0.1 mM PMSF at pH 7.5. The suspension is homogenized in a glass-glass Potter-Elvejhem. The homogenate is then centrifuged at 105 000 *g* for 60 min. The 105 000 *g* supernatant is stored at –75ºC until injection on the HPLC column.

Isolation of PDEs

The cytosolic fraction from rat ventricles (5–8 mg of protein) is loaded at the rate of 1 ml/min on a Mono Q HPLC column which has been previously equilibrated with buffer A (50 mM Tris-HCl, 2 mM EDTA, 14 mM 2-mercaptoethanol, 0.1 mM PMSF, pH 7.5). Under these conditions, greater than 95% of the PDE activity is bound to the column. PDE activity is eluted at a flow rate of 1 ml/min using the following step by step and linear gradients of NaCl in buffer A: 25 ml of 0.16 M NaCl, 20 ml of 0.23 M NaCl, 30 ml from 0.23 to 0.29 M NaCl, 15 ml of 0.29 M NaCl, 30 ml from 0.29 to 0.50 M NaCl. The separation is done at 4 °C. Fractions of 1 ml are collected and stored at –75 °C in the presence of 20% glycerol. The fractions are tested for PDE activity, and the peaks containing the different isoenzymes are identified. Fractions containing preferentially one isoenzyme are pooled.

PDE assay

PDE activity is assayed by a two-step radioisotopic procedure according to Thompson et al. (1974), Boudreau and Drummond (1975), Prigent et al. (1981). cAMP-PDE and cGMP-PDE activities are measured with a substrate concentration of 0.25 µM. To evaluate the cGMP-stimulated PDE activity, assays are performed with 5 µM cAMP in the absence or presence of 5 µM cGMP. Xanthine derivates are dissolved in DMSO. The stock solutions are appropriately diluted with 40 mM Tris-HCl buffer so that the final DMSO concentration in the PDE assay does not exceed 1%. At this concentration, DMSO has no significant effect on the PDE activity of any of the fractions. The inhibitory potency of the xanthine derivates is examined on each separated isoform.

EVALUATION

The IC_{50} values (concentration of a drug which inhibit 50% of the enzymatic activity) are calculated by plotting the percentage of residual enzymatic activity versus the logarithmic concentration of the drug. Confidence limits (95%) for the IC_{50} values are determined by linear regression analysis.

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A.1.1.21 Stimulation of heart membrane adenylate cyclase

PURPOSE AND RATIONALE

Metzger and Lindner (1981) discovered that the positive inotropic and vasodilatory effects of forskolin were correlated with the stimulation of adenylate cyclase and cAMP-dependent protein kinase. Subsequent studies by Seamon (1981) demonstrated that forskolin, unlike hormones, guanine nucleotides, fluoride or cholera toxin could stimulate cyclase activity in the absence of the guanine nucleotide regulatory protein. Since those reports, hundreds of papers have been published on the effects of forskolin in numerous mammalian organ and cell systems. Several comprehensive review articles have also been published (Seamon and Daly 1981, 1983; Daly 1984).

While forskolin has proven to be an invaluable research tool for investigations of adenylate cyclase systems (Salomon et al. 1974; Seamon et al. 1981, 1983), reports on its effects on cardiovascular (Lindner et al. 1978), pulmonary (Chang et al. 1984) and ocular physiology (Caprioli and Sears 1983; Caprioli 1985) suggest a therapeutic potential (Seamon 1984) as well.

Described here is an *in vitro* assay which can be used to compare the potency of forskolin, forskolin analogs or other direct or indirect adenylate cyclase activators for the stimulation of adenylate cyclase in heart membranes. The purpose of this assay is to determine and compare the potency of direct or indirect activators of adenylate cyclase for an ability to stimulate heart membrane adenylate cyclase *in vitro*.

PROCEDURE

The hearts of the Wistar rat, Hartley guinea pig, golden Syrian hamster or cardiomyopathic hamster (CHF-146) are used as a source of adenylate cyclase for this assay.

Reagents

1. 0.5 M Tris buffer, pH 7.4
2. 0.05 M Tris buffer, pH 7.4, containing 0.1 M $CaCl_2$
3. Tris buffer mixture

   0.05 M Tris buffer, containing 1 mM IMBX (isobutylmethyl-xanthine), 0.2 mM EGTA, 5 mM $MgCl_2$, 0.5 mM ATP ($Na_2ATP \times 3\ H_2O$) and 20 mM creatine phosphate (Na_2 Creatine-$PO_4 \times 5\ H_2O$) (final concentrations in the incubation media).
4. Creatine phosphokinase (ATP:Creatine N-Phosphotransferase, EC 2.73.2), type I, from rabbit muscle is obtained from Sigma Chemical Co.

   The concentration of enzyme in the incubation media is 40 U/ml.
5. Test compounds

   For most assays, a 30 mM stock solution is made up in a suitable solvent (ethanol, ethyl acetate or DMSO for most forskolin analogs). Serial dilutions are made such that the final concentration in the assay ranges from 3×10^{-4} to 3×10^{-7} M. The concentration of vehicle in the assay is 1%.

Tissue preparation

One entire heart from rat, guinea pig or hamster is dissected, weighed and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer, pH 7.4 containing 0.1 mM $CaCl_2$ (reagent 2) using a Brinkman Polytron (setting 7 for 15 s). The homogenate is centrifuged at 10 000 *g* for 20 min at 4 °C. The resulting pellet is resuspended in 50 volumes of homogenizing buffer and recentrifuged as before. The supernatant of this spin is discarded and the resulting pellet is finally resuspended in 10 volumes of the homogenizing buffer, for rat and hamster and 60 volumes for guinea pig tissue. This final preparation is filtered through a thin layer of gauze and kept on ice until used in the assay. The protein concentration is approximately 250–350 mg/ml.

Protein concentrations from an aliquot of the tissue suspension are determined on the day of the experiment by the method of Bradford (1976) using the BioRad assay kit.

Assay

300 µl Tris buffer mixture, pH 7.4 (reagent 3)
50 µl creatine phosphokinase
5 µl vehicle or appropriate concentration of test drug
95 µl H_2O
50 µl tissue suspension

The tubes are incubated for 5 min at 37 °C and the reaction is then stopped by placing the tubes into a boiling water bath for 4 min. The tubes are then centrifuged at 1 000 *g* for 10 min and cAMP levels determined in a 15 µl aliquot of the supernatant using a radioimmuno-assay kit (Code TRK432) obtained from Amersham according to the manufacturer's protocol.

Test principle: The method is based on the competition between unlabeled cAMP and a fixed quantity of ^{3}H-labelled cAMP for binding to a protein which has high specificity and affinity for cyclic AMP. The amount of labelled cAMP–protein complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. The concentration of cAMP in the unknown is determined by comparison with a linear standard curve.

EVALUATION

The data are expressed as pmol cAMP/mg protein/min and dose-response stimulation curves are subjected to logic analysis to determine the concentration of compound which exhibits 50% of maximal stimulation (ED_{50}).

The β-blocking activities of compounds can be determined by their activities, by which they counteract the isoproterenol and GTP induced stimulation of rat heart membrane bound adenylate cyclase (Greenslade et al. 1979).

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A.1.1.22 ^{3}H-Forskolin binding assay

PURPOSE AND RATIONALE

This assay is used to identify compounds which demonstrate high affinity [nM] for association with forskolin binding sites *in vitro* as a preliminary screen in conjunction with stimulation of adenylate cyclase to determine the potential for cardiac chronotropic and inotropic and other effects of forskolin. Guinea pig heart tissue and rat brain tissue are used as sources of binding assays.

PROCEDURE FOR GUINEA PIG HEART TISSUE

A. Reagents

1. 0.05 M Tris-HCl buffer, pH 7.4
2. 0.05 M Tris-HCl, pH 8.0 containing 1 mM EGTA, 1 mM $MgCl_2$ and 0.32 M sucrose

3. [^{3}H]-Forskolin (specific activity 31.6 Ci/mmol) is obtained from New England Nuclear.

   Final concentration in the assay is approximately 15 nM.

4. Forskolin and test compounds

   Forskolin and forskolin analogs are diluted to 40 µM. A 100 µl addition of this solution to the final incubate of 200 µl tissue and 100 µl ^{3}H-forskolin results in a final concentration of 10^{-5} M.

   Appropriate serial dilutions of this stock solution are made such that the final concentrations in the assay range from 10^{-5} to 10^{-11} M with each concentration being done in triplicate.

B. Tissue preparation

Male Hartley guinea pigs (300–350 g) are sacrificed by decapitation. The heart is immediately removed, weighed, rinsed, diced and homogenized in 10 volumes of ice-cold 0.05 M buffer(reagent 2) using a Polytron homogenizer (setting 10, 30 s). The resulting homogenate is centrifuged at 12 000 *g* for 15 min at 4 °C. The clear supernatant of this spin is discarded, the remaining pellet (P_2) is resuspended in 5 volumes of the same ice-cold buffer and rehomogenized. This final suspension (approximately 0.8–1.0 mg protein/ml) is kept on ice until use. Protein concentrations are estimated according to the method of Lowry et al. (1951).

C. Binding assay

To generate a dose-response inhibition curve, ^{3}H-forskolin binding is performed according to the method of Seamon et al. (1984) with minor modifications.

200 µl tissue suspension
100 µl ^{3}H-forskolin
100 µl appropriate concentration of forskolin or forskolin analog, or buffer

Tubes are incubated at 30 °C for 10 min. The incubate is then diluted with 5 ml of ice-cold 0.05 M Tris-HCl buffer, pH 7.4 (reagent 1) and immediately vacuum filtered through glass fiber filters (Whatman GF/B) by using a Brandel Cell Harvester. The filters are rapidly washed 3 times with 5 ml aliquots of Tris-HCl buffer (reagent 1), added to 10 ml scintillation cocktail and analyzed for radioactivity.

PROCEDURE FOR RAT BRAIN TISSUE

A. Reagents

1. 0.32 M sucrose buffer
2. 0.05 M Tris-HCl buffer, pH 7.5
3. [^{3}H]-Forskolin (specific activity 31.6 Ci/mmol) is obtained from New England Nuclear.

   Final concentration in the assay is approximately 10 nM.

4. Forskolin and test compounds

   Forskolin and forskolin analogs are diluted to 40 µM. A 100 µl addition of this solution to the final incubate of 200 µl tissue and 100 µl ^{3}H-forskolin results in a final concentration of 10^{-5} M.

   Appropriate serial dilutions of this stock solution are made such that the final concentrations in the assay range from 10^{-5} to 10^{-11} M with each concentration being done in triplicate.

B. Tissue preparation

Male Sprague-Dawley rats (200–250 g) are sacrificed by decapitation. The brain is rapidly removed and dissected on ice. Striata are homogenized in 50 volumes of ice-cold 0.32 M sucrose buffer (reagent 1) using a Polytron homogenizer (setting 7, 15 s). The resulting homogenate is centrifuged at 1 000 *g* for 10 min at 0–4 °C. The supernatant is retained and recentrifuged at 20 000 *g* for 10 min at 0–4 °C. The clear supernatant of this spin is discarded and the remaining pellet (P_2) is resuspended in ice-cold Tris-HCl buffer, pH 7.5 such that the final protein concentration is approximately 3.0–4.0 mg/ml. Protein concentrations are estimated according to the method of Lowry et al. (1951).

C. Binding assay

To generate a dose-response inhibition curve, ^{3}H-forskolin binding is performed according to the method of Seamon et al. (1984) with minor modifications.

200 µl tissue suspension
100 µl ^{3}H-forskolin
100 µl appropriate concentration of forskolin or forskolin analog or buffer

Tubes are incubated at 23 °C for 60 min. The incubate is then diluted with 5 ml of ice-cold 0.05 M Tris-HCl buffer, pH 7.4 (reagent 2) and immediately vacuum filtered through glass fiber filters (Whatman GF/B) by using a Brandel Cell Harvester. The filters are rapidly washed three times with 5 ml aliquots of Tris-HCl buffer (reagent 2), added to 10 ml scintillation cocktail and analyzed for radioactivity.

EVALUATION

Specific binding is defined as the difference between binding of ^{3}H-forskolin in the absence and presence of 10 µM forskolin and represents 85–90% of total binding at 10 nM ^{3}H-forskolin.

Results of the dose-response inhibition curves are analyzed by determining the concentration of competing compound which inhibits 50% of the ^{3}H-forskolin binding sites (IC_{50}). This value is determined by computer-derived log-probit analysis.

The activity of various forskolin analogs are based on IC_{50} values and are categorized as follows:

	IC_{50} [M]
0 = Not determined	
1 = No activity	$>10^{-5}$
2 = Slight activity	10^{-5}–10^{-6}
3 = Moderate activity	10^{-6}–10^{-7}
4 = Marked activity	$<10^{-7}$

To compare the activity of various compounds from experiment to experiment, an inhibition constant (K_I) is determined as described by Cheng and Prusoff (1973). The K_I is determined from the equation:

$$K_I = IC_{50} / 1 + LC / K_D$$

IC_{50} = concentration of competing compound which inhibits 50% of the ^{3}H-forskolin binding sites
LC = determined ^{3}H-forskolin concentration (approximately 10 nM)
K_D = dissociation of affinity constant for ^{3}H-forskolin determined previously to be approximately 13.4 nM for rat striatum and 196 nM for guinea pig heart

Standard data:
Binding inhibition values for forskolin

	Striatum	Heart
IC_{50}	43.1 ±4.9 nM	34.2 ±5.0 nM
K_I	26.0 ±3.1 nM	31.6 ±4.6 nM

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A.1.1.23 Patch clamp technique[2]

PURPOSE AND RATIONALE

The introduction of the patch clamp technique (Neher and Sakmann 1976) revolutionized the study of cellular physiology by providing a high-resolution method of observing the function of individual ionic channels in a variety of normal and pathological cell types. By the use of variations of the basic recording methodology, cellular function and regulation can be studied at a molecular level by observing currents through individual ionic channels (Liem et al. 1995; Sakmann and Neher 1995).

The most commonly used method is called the "on-cell" or "cell-attached" configuration, because ion channels can be recorded on an intact cell (Jackson 1993). This mode is well suited for investigation of ion channels, which are activated by hormonal stimulation and triggered by intracellular second messengers.

Another versatile mode is the "cell-excised" configuration (Hamill 1993). It is obtained by suddenly removing the patch-pipette from the cell, so that the membrane patch is pulled off the cell. This mode easily allows to expose the channel proteins to drugs by changing the bath solution. The single channel currents are recorded on a video-tape and are analyzed off-line by a computer system. Various parameters are evaluated, such as the single channel conductance, open-and closed-times of the channel, and the open-state probability, which is the percentage of time the channel stays in its open state.

Besides these modes, which enable the recording of single channel currents, it is also possible to measure the current flowing through the entire cell. This "whole-cell mode" is obtained by rupturing the membrane patch in the cell-attached mode (Hamill et al. 1981; Dietzel et al. 1993). This is achieved by applying suction to the interior of the patch-pipette. The "whole-cell mode" not only allows to record the electrical current, but also to measure the cell potential. Moreover, the cell interior is dialyzed by the electrolyte solution filled into the patch-pipette.

The fabrication of patch clamp pipettes has been described by Sakmann and Neher 1983; Corey and Stevens 1983; Cavalié et al. 1993).

Variations of the patch-clamp technique have been used for studying neurotransmitter transduction mechanisms (Smith 1995).

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A.1.1.23.1 Patch clamp technique in isolated cardiac myocytes

PURPOSE AND RATIONALE

The generation of an action potential in heart muscle cells depends on the opening and closing of ion-selective channels in the plasma membrane. The patch-clamp technique enables the investigation of drug interactions with ion-channel forming proteins on the molecular level.

PROCEDURE

Isolated cells from rat ventricular muscle are prepared as described by Yazawa et al. (1990). Male Wistar rats are sacrificed by cervical dislocation. Hearts are dissected and mounted on a Langendorff-type apparatus and perfused first with Tyrode solution (in mM: 143 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.25 NaH_2PO_4, 5 HEPES, pH adjusted to 7.4 with NaOH) at 37 °C for 3 min at a hydrostatic pressure of 60–70 cm H_2O, then with nominally Ca^{2+}-free Tyrode solution (no Ca^{2+} is added) for 5–7 min, and finally with nominally Ca^{2+}-free Tyrode solution containing 0.12–0.2 mg/ml collagenase (Sigma, type I). After 15–20 min of collagenase treatment, the heart is now soft and is washed with storage solution (in mM: 70 KOH, 50 L-glutamic acid, 40 KCl, 20 taurin, 20 KH_2PO_4, 3 $MgCl_2$, 10 glucose, 10 HEPES, 0.5 EGTA, pH adjusted to 7.4 with KOH). The ventricles are cut into pieces (about 5 × 5 mm) and poured into a beaker. The myocytes are dispersed by gently shaking the beaker and filtration through a nylon mesh (365 µm). Then, the myocytes are washed twice by centrifugation at 600–1 000 rpm (about 90 *g*) for 5 min and kept at 4 °C. The rod shape of the cell and the clear striations of sarcomeres are important criteria for selecting viable cells for the assay. Experiments are performed at 35–37 °C.

For investigation with the patch-clamp technique (Neher and Sakmann 1976; Hamill et al. 1981), the isolated cells are placed into a thermostat-controlled chamber, mounted on the stage of an inverted microscope equipped with differential interference contrast optics. Under optical control (magnification 400×) a glass micro-pipette, having a tip opening of about 1 µm, is placed onto the cell. The patch-pipettes are fabricated from borosilicate glass tubes (outer diameter 1.5 mm, inner diameter 0.9 mm) by means of an electrically heated puller. In order to prevent damage of the cell membrane, the tip of the micro-pipette is fire polished, by moving a heated platinum wire close to the tip. The patch-pipette is filled with either high NaCl or KCl solution and is mounted on a micro manipulator. A chlorided silver wire connects the pipette solution to the head stage of an electronical amplifier. A second chlorided silver wire is inserted into the bath and serves a ground electrode.

After having contact with the cell membrane, a slight negative pressure is applied to the inside of the patch-pipette by means of a syringe. Consequently, a small patch of membrane is slightly pulled into the opening of the micro pipette and a close contact between glass and membrane is formed, leading to an increase of the electrical input resistance into the gigaohm range (about 10^{10} Ohm). This high input resistance enables the recording of small electrical currents in the range of Picosiemens (10^{-12} S), which are flowing through channel-forming proteins situated in the membrane patch. The electrical current is driven by applying an electrical potential across the membrane patch, and/or by establishing an appropriated chemical gradient for the respective ion species.

The patch clamp methods allows to investigate the interaction of drugs with all ion channels involved in the functioning of the heart muscle cell (K^+, Na^+, Ca^{2+} and eventually Cl^- channels). Moreover, the different types of K^+ channels existing in cardiomyocytes can be distinguished by their different single-channel characteristics or by appropriate voltage-pulse protocols in the whole-cell mode.

EVALUATION

Concentration-response curves of drugs which either inhibit or activate ion channels can be recorded either on the single channel level or by measuring the whole-cell current. IC_{50} and EC_{50} values (50% inhibition or activation, respectively) can be obtained with both methods.

MODIFICATIONS OF THE METHOD

The patch-clamp technique has been used for evaluation of anti-arrhythmic agents (Bennett et al. 1987; Anno and Hondeghem 1990; Gwilt et al. 1991).

Gögelein et al. (1998) used isolated ventricular myocytes form guinea pigs to study a cardioselective inhibitor of the ATP-sensitive potassium channel.

Multiple types of calcium channels have been identified by patch clamp experiments (Tsien et al. 1988).

The effects of potassium channel openers have been measured (Terzic et al. 1994; Kon et al. 1994).

Ryttsén et al. (2000) characterized electroporation of single NG108-15 cells with carbon-fiber microelectrodes by patch-clamp recordings and fluorescence microscopy.

Monyer and Lambolez (1995) reviewed the molecular biology and physiology at the single-cell level discussing the value of the polymerase chain reaction at the single cell level and the use of patch pipettes to collect the contents of a single cell on which the reverse transcription is performed.

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A.1.1.23.2 Voltage clamp studies on sodium channels

PURPOSE AND RATIONALE

The epithelial Na^+ channel plays an important role in epithelial Na^+ absorption in the distal colon, urinary bladder, salivary and sweat ducts, respiratory tract and, most importantly in the distal tubules of the kidney (Catterall 1986; Palmer 1992). Regulation of this epithelial Na^+ channel has a major impact on Na^+ balance, blood volume and blood pressure. Inhibition of epithelial Na^+-channel expression is used for the treatment of hypertension (Endou and Hosoyamada 1995). Busch et al. (1995) studied the blockade of epithelial Na^+ channels by triamterenes using two microelectrodes voltage clamp experiments in *Xenopus* oocytes expressing the three homologous subunits (α, β and γ) of the rat epithelial Na^+ channel (rENaC).

PROCEDURE

Xenopus laevis oocytes are injected with the appropriate cRNA encoding for the α, β and γ-subunits (Canessa et al. 1993) of the rat epithelial Na^+ channel (rENaC). The cRNA for the wild-type α-subunit and its deletion mutant Δ278–273, is always co-injected with an equal amount of β-and γ-subunit cRNA (10 ng/oocyte).

Two to 8 days after cRNA injection, the two-microelectrode voltage clamp is used to record currents from *Xenopus* oocytes. Recordings are performed at 22 °C using an Geneclamp amplifier (Axon Instruments, Foster City, CA, USA), and MacLab D/A converter and software for data acquisition and analysis (AD Instruments, Castle Hill Australia). The ND 96 solution (control) contains (mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.0). In some experiments, Na^+ is replaced by *N*-methyl-D-glucamine (NMDG) solution. The microelectrodes are filled with 3 M KCl solution and have resistances in the range 0.5–0.9 MΩ. Chemicals (e.g., triamterene as standard) are added in concentrations between 0.2 to 100 μM). The amplitude of the induced currents varies considerably, depending on the day of channel expression and the batch of oocytes. The mutant channel induces considerably smaller currents than the wild-type channel. The total Na^+ current amplitude is determined at least once for each experimental day by superfusion with NMDG solution, or with 3 μM or 5 μM amiloride solution at the beginning and at the end of each set of experiments.

EVALUATION

Data are presented as means ±SEM. A paired Student's *t*-test is used. The level of statistical significance is set at $P < 0.05$.

MODIFICATIONS OF THE METHOD

Nawada et al. (1995) studied the effects of a sodium-, calcium, and potassium-antagonistic agent on the sodium current by the whole cell voltage clamp technique (tip resistance = 5 MΩ[Na]$_i$ and [Na]$_o$ 10 mmol/l at 20 °C) in guinea-pig isolated ventricular cells.

Sunami and Hiraoka (1996) studied the mechanism of cardiac Na^+ channel block by a charged class I antiarrhythmic agent, in guinea pig ventricular myocytes using patch-clamp techniques of whole-cell, cell-attached and inside-out configurations.

Erdõ et al. (1996) compared the effects of *Vinca* derivatives on voltage-gated Na^+ channels in cultured cells from rat embryonic cerebral cortex. Effects on Na^+ currents were measured applying voltage steps (20 ms duration) to –10 mV from a holding potential of –70 mV every 20 s. Steady state inactivation curves were obtained by clamping the membrane at one of a series of 15-s pre-pulse potentials, followed 1 ms later by a 20-ms test pulse to –10 mV.

Ragsdal et al. (1993) examined the actions of a Na^+ channel blocker in whole-cell voltage clamp recordings from Chinese hamster ovary cells transfected with a cDNA encoding the rat brain type IIA Na^+ channel and from dissociated rat brain neurons.

Taglialatela et al. (1996) studied cloned voltage-dependent Na^+ currents expressed in *Xenopus* oocytes upon injection of the cRNA encoding α-subunits from human and rat brain.

Eller et al. (2000) measured the effects of a calcium antagonist on inward Na^+ currents (I_{Na}) in GH3-cells with the whole-cell configuration of the patch clamp technique. I_{Na} was recorded after depolarization from a holding potential of –80 mV to a test potential of +5 mV. Initial 'tonic' block (resting state-dependent block) was defined as peak I_{Na} inhibition during the first pulse 2 min after drug application as compared with I_{Na} in the absence of drug. 'Use- (frequency-)-dependent' block of I_{Na} was measured during trains of 5 or 50 ms test pulses (3 Hz) applied from –80 mV to a test potential of +5 mV after a 2-min equilibrium period in the drug-containing solution. Use-dependent block was expressed as the percent decrease of peak I_{Na} during the last pulse of the train as compared with I_{Na} during the first pulse.

Khalifa et al. (1999) characterized the effects of an antidepressant agent on the fast inward current (I_{Na}) in isolated guinea pig ventricular myocytes. Currents were recorded in the whole-cell configuration of the patch-clamp technique in the presence of Ca^{2+} and K^+ channel blockers.

Haeseler et al. (1999) measured the effects of 4-chloro-m-cresol, a preservative added to a wide variety of drugs, on heterologously expressed wild type, Paramyotonia congenita (R1448H) and hyperkalemic periodic paralysis (M1360V) mutant α-subunits of human muscle sodium channels in whole-cell and inside-out voltage clamp experiments.

Song et al. (2000) studied the effects of *N*-ethylmaleimide, an alkylating agent to protein sulfhydryl groups, on tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium channels in rat dorsal root neurons using the whole cell configuration of the patch-clamp technique. Rats at the age of 2–6 days were anesthetized with isofluorane and the spinal cord was removed and cut longitudinally. Dorsal root ganglia were plucked from the area between the vertebrae of the spinal column, and incubated in phosphate-buffered saline solution containing 2.5 mg/ml trypsin at 37 °C for 30 min. After enzyme treatment, ganglia were rinsed with Dulbecco's Modified Eagle Medium supplemented with 10% horse serum. Single cells were mechanically dissociated by trituration with a fire-polished Pasteur pipette and plated on poly-L-lysine-coated glass coverslips. Cells attached to the coverslips were transferred into a recording chamber on the stage of an inverted microscope. Ionic currents were recorded under voltage-clamp conditions by the whole-cell patch-clamp technique. The solution of the pipette contained (in mM): CsCl 125, NaF 20, HEPES 5, EGTA 5. The pH was adjusted to 7.2 with CsOH and the osmolarity was 279 mosM/l on average. The external solution contained (in mM): NaCl 50, choline chloride 90, tetramethylammonium chloride 20, D-glucose 5, HEPES 5, $MgCl_2$ 1, $CaCl_2$ 1. Lanthanum ($LaCl_3$, 10 μM) was used to block calcium channel current. The solution was adjusted to pH 7.4 with tetramethylammonium hydroxide and the osmolarity was 304 mosM/l on average. An Ag–AgCl pellet/3 M KCl-agar bridge was used for the reference electrode. Membrane currents were recorded using an Axopatch-1D amplifier. Signals were digitized by a 12-bit analog-to-digital interface, filtered with a lowpass Bessel filter at 5 kHz and sampled at 50 kHz using pCLAMP6 software (Axon Instruments) on an IBM-compatible PC. Series resistance was compensated 60–70%. Capacitative and leakage currents were subtracted by using a P + P/4 procedure (Bezanilla and Armstrong 1977). The liquid junction potential between internal and external solution was on average –1.7 mV. TTX (100 nM) was used to separate TTX-R sodium currents from TTX-S sodium currents. For the study of TTX-S sodium channels, cells that expressed only TTX-S sodium channels were used. TTX-S sodium channels were completely inactivated within 2 ms when currents were evoked by depolarizing steps to 0 mV, while TTX-R sodium channels persisted for more than 20 ms. The difference in kinetics was used to identify the type of sodium current.

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A.1.1.23.3 Voltage clamp studies on potassium channels

PURPOSE AND RATIONALE

Potassium channels represent a very large and diverse collection of membrane proteins which participate in important cellular functions regulating neuronal and cardial electrical patterns, release of neurotransmitters, muscle contractility, hormone secretion, secretion of fluids, and modulation of signal transduction pathways. Main categories of potassium channels are gated by voltage or an increase of intracellular calcium concentration (Escande and Henry 1993; Kaczorowski and Garcia 1999; Alexander et al. 2001). For ATP-sensitive potassium channels see Sect. K.6.1.9.

The delayed outward potassium current in heart muscle cells of several species in made up of a rapidly (I_{Kr}) and a slowly (I_{Ks}) activating component (Sanguinetti and Jurkiewicz 1990; Wang et al. 1994; Gintant 1996; Lei and Brown 1996; Carmeliet and Mubagawa 1998). Several potent and selective blockers for the I_{Kr} channel have been shown to prolong the effective refractory period, but have a reverse rate-dependent activity with both normal and elevated extracellular potassium concentrations (Colatsky et al. 1990). Inhibitors of the slow component I_{Ks} were developed in order to circumvent the negative rate dependence of I_{Kr} channel blockers on the effective refractory period (Busch et al. 1996; Suessbrich et al. 1996, 1997; Bosch et al. 1998). Gögelein et al. (2000) studied the effects of a potent inhibitor of I_{Ks} channels in *Xenopus* oocytes and guinea pig ventricular myocytes.

PROCEDURE

Studies in *Xenopus* oocytes are performed with the two microelectrode voltage clamp method. For isolation of the oocytes, the toads are anesthetized using a 1 g/l solution of 3-aminobenzoic acid ethyl ester and placed on ice. A small incision is made to retrieve sacs of oocytes and is subsequently closed with absorbable surgical suture. On waking up, the toads are placed back into the aquarium. The ovaries are cut up into small pieces and the oocytes are washed in Ca^{2+}-free Or-2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES; pH 7.4) and subsequently collagenized in Or-2 containing collagenase A (1 mg/ml, Worthington, type II) until follicles are not longer detectable on the oocyte surface. The oocytes are stored at 18 °C in recording solution ND-96 (NaCl 96 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 5 mM, pH 7.4) with additions of sodium pyruvate (275 mg/l), theophylline (90 mg/l) and gentamycin (50 mg/l).

For electrophysiological recordings, the two-microelectrode voltage-clamp configuration is used to record ion currents from *Xenopus* oocytes. Injection of cRNA is performed according to Methfessel et al. (1986), Golding (1992). Oocytes are injected individually with cRNA encoding for the human protein minK, guinea pig Kir2.1, human H*erg*, human Kv1.5, mouse Kv1.3, or human HNC2. In the case of minK the functional potassium channel is a heteromultimer composed of the endogenous (*Xenopus*) KvLQT1 and the injected human

minK. This heteromultimeric potassium current in then called I_{Ks} (Barhanin et al. 1996; Sanguinetti et al. 1996).

The electrophysiological recordings are performed at room temperature, using a Geneclamp amplifier (Axon Instruments, Foster City, CA, USA), and MacLab D/A converter. The amplitudes of the recorded currents are measured at the end of the test voltage steps. The control ND-96 solution contains (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, (pH 7.4). To amplify the inward potassium current through Kir2.1 and HNC2, the external potassium concentration is raised to 10 mM KCl and the NaCl concentration lowered to 88 mM (ND-88). The microelectrodes are filled with 3 M KCl and have a resistance between 0.5 MW and 1 MW. During the recordings the oocytes are continuously perfused with ND-96 (or ND-88 in the case of Kir2.1 and HCN2). The test compounds are dissolved in DMSO and added to the buffer ND-96 or ND-88. The current amplitude is determined after 5 min of wash-in time.

For the isolation of *ventricular myocytes*, guinea pigs (weight about 400 g) or Sprague-Dawley rats of either sex are sacrificed by cervical dislocation. The hearts are dissected and perfused retrogradely via the aorta at 37°C: first with nominally Ca^{2+}-free Tyrode solution (in mmol/l): 143 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 0.25 NaH_2PO_4, 10 glucose, 5 HEPES, pH 7.2, then with Tyrode solution containing 20 mmol/l Ca^{2+} and 3 mg/ml collagenase type CLS II (Biochrom, Berlin Germany). After 5–10 min collagenase treatment the ventricles are cut up into small pieces in the storage solution (in mmol/l): 50 L-glutamic acid mono-potassium salt, 40 KCl, 20 taurine, 20 KH_2PO_4, 1 $MgCl_2$, 10 glucose, 0.2 EGTA, pH 7.2. The myocytes are then dispersed by gentle shaking followed by filtration through a nylon mesh (365 mm). The cells are finally washed twice by centrifugation at 90*g* for 5 min and kept in the storage solution at room temperature.

Whole cell currents are recorded in the tight-seal whole-cell mode of the patch-clamp technique, using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany). Patch pipettes are pulled from borosilicate glass capillaries (wall thickness 0.3 mm, outer diameter 1.5 mm) and their tips are fire-polished. Series resistance is in the range of 1–10 MW and 50% compensated by means of the EPC's compensation circuit.

The I_{Ks}, I_{Kr}, I_{K1} and currents are investigated in guinea pig ventricular myocytes. The voltage pulses for recording the current components are as follows: I_{Ks} current: holding potential –80 mV to –50 mV (200 ms) to +60 mV (3 s) to –40 mV (2 s) to –80 mV; I_{Kr} current: holding potential –80 mV to –50 mV (200 ms) to –10 mV (3 s) to –40 mV (2 s) to –80 mV. I_{Kr} is evaluated as the tail current evoked by a voltage pulse from –10 mV to –40 mV; I_{K1}: holding potential –80 mV to –120 mV (200 ms) to –80 mV. In order to suppress the L-type Ca^{2+} current, 5 mmol/l nifedipine is added to the bath solution.

EVALUATION

All average data are presented as means ±SEM. Student's *t*-test is used to determine the significance of paired observations. Differences are considered as significant at $P < 005$.

MODIFICATIONS OF THE METHOD

Using the whole-cell configuration of the patch-clamp technique, Grissmer et al. (1994) analyzed the biophysical and pharmacological properties of five cloned voltage-gated K^+ channels stably expressed in mammalian cell lines.

Sanchez-Chapula (1999) studied the block of the transient outward K^+ channel (I_{to}) by disopyramide in isolated rat ventricular myocytes using whole-cell patch-clamp techniques.

Using the patch clamp technique, Cao et al. (2001) investigated the effects of a centrally acting muscle relaxant and structurally related compounds on recombinant small-conductance Ca^{2+}-activated K^+ channels (rSK2 channels) in HEK mammalian cells.

Tagliatela et al. (2000) discussed the block of the K^+ channels encoded by the human *ether-á-go-go-related* gene (HERG), termed $K_{V(r)}$, which are the molecular determinants of the rapid component of the cardiac repolarizing current $I_{K(Vr)}$, involved in the cardiotoxic potential and CNS effects of first-generation antihistamines and may be therapeutic targets for antiarrhythmic agents (Vandenberg et al. 2001).

Chabbert et al. (2001) investigated the nature and electrophysiological properties of Ca^{2+}-independent depolarization-activated potassium currents in acutely isolated mouse vestibular neurons using the whole-cell configuration of the patch-clamp technique. Three types of currents were identified.

Caballero et al. (2001) analyzed the effects of angiotensin II type 1 receptor antagonists on cloned potassium channels (hKv1.5, HERG, KvLQT1+minK, Kv4.3) expressed in *Ltk*$^-$ or Chinese hamster ovary cells using the patch-clamp technique.

Longobardo et al. (2000) studied the effects of a quaternary bupivacaine derivative on delayed rectifier K^+ currents stably expressed in *Ltk*$^-$ cells using the whole-cell configuration of the patch-clamp technique.

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A.1.1.23.4 Voltage clamp studies on calcium channels

PURPOSE AND RATIONALE

Calcium influx through voltage-gated Ca^{2+} channels mediates a range of cytoplasmatic responses, including muscle contraction, release of neurotransmitters, Ca^{2+} dependent gene transcription and the regulation of neuronal excitability has been reviewed by several authors (Augustine et al. 1987; Bean 1989; Miller 1987; Zamponi 1997; Snutch et al. 2001). In addition to their normal physiological function, Ca^{2+} channels as calcium antagonists are also implicated in a number of human disorders (see also A.4.0.1).

Using patch clamp techniques, the structure and regulation of voltage-gated Ca^{2+} channels has been studied by many authors (Sculptoreanu et al. 1993; Peterson et al. 1997; Catterall 2000).

Berjukow et al. (2000) analyzed the role of the inactivated channel conformation in molecular mechanism of Ca^{2+} channel block by a dihydropyridine derivative in L-type channel constructs and mutants in *Xenopus* oocytes and described the electrophysiological evaluation.

PROCEDURE

Inward barium currents (I_{Ba}) are studied with two microelectrode voltage-clamp of *Xenopus* oocytes 2–7 days after microinjection of approximately equimolar cRNA mixtures of constructs of L-channel mutants. All experiments are carried out at room temperature in a bath solution with the following composition: 40 mM $Ba(OH)_2$, 50 mM NaOH, 5 mM HEPES,

2 mM CsOH (pH adjusted to 7.4 with methanesulfonic acid). Voltage recording and current injecting microelectrodes are filled with 2.8 M CsCl, 0.2 M CsOH, 10 mM EGTA, 10 mM HEPES (pH 7.4) with resistances of 0.3–2 MΩ. Resting channel block is estimated as peak I_{Ba} inhibition during 100-ms test pulses from −80 to 20 mV at a frequency of 0.033 Hz until steady state is reached. The dose response curves of I_{Ba} inhibition were fitted using the Hill equation:

$$\frac{I_{Ba,drug}}{I_{Ba,control}}(\%) = \frac{100 - A}{1 + \left(\frac{C}{IC_{50}}\right)^{nH}} + A$$

where IC_{50} is the concentration at which I_{Ba} inhibition is half-maximal, C is the applied drug concentration, A is the fraction of I_{Ba} that is not blocked, and nH is the Hill coefficient.

Recovery from inactivation is studied at a holding potential of −80 mV after depolarizing Ca^{2+} channels during a 3-s prepulse to 20 mV by applying 30-ms test pulses (to 20 mV) at various time intervals after the conditioning prepulse. Peak I_{Ba} values are normalized to the peak current measured during the prepulse, and the time course of I_{Ba} recovery from inactivation is fitted to a mono- or biexponential function

$$I_{Ba,recovery} = A \times \exp\left(\frac{-t}{\tau_{fast}}\right) + B \times \exp\left(\frac{-t}{\tau_{slow}}\right) + C$$

Voltage dependence of inactivation under quasi-steady state conditions is measured using a multi step protocol to account for run-down (less than 10%). A control test pulse (50 ms to 20 mV) is followed by a 1.5-s step to−100 mV followed by a 30-s conditioning step, a 4-ms step to −100 mV, and a subsequent test pulse to 20 mV (corresponding to the peak potential of the *I-V* curves).

Inactivation during the 30 s conditioning pulse is calculated as follows.

$$I_{Ba,inactivation} = \frac{I_{Ba,test}(20\,mV)}{I_{Ba,control}(20\,mV)}$$

The pulse sequence is applied every 3 min from a holding potential of −100 mV. Inactivation curves are drawn according to the following Boltzmann equation.

$$I_{Ba,inactivation} = I_{SS} + (1 - I_{SS})\left(1 + \exp\left(\frac{V - V_{0.5}}{k}\right)\right)$$

where V is the membrane potential, $V_{0.5}$ is the midpoint voltage, k is the slope factor, and I_{SS} is the fraction of non inactivating current.

Steady state inactivation of the mutate channels at −80 mV is estimated by shifting the membrane holding potential from −80 to −100 mV. Subsequent monitoring of the corresponding changes in I_{Ba} amplitudes until steady state reveals the fraction of Ca^{2+} channels in the inactivated state at −80 mV. Steady state inactivation of different L-type channel constructs at −30 mV is estimated by fitting time course of current inactivation to a biexponential function.

The I_{Ba} inactivation time constants are estimated by fitting the I_{Ba} decay to a mono- or biexponential function.

EVALUATION

Data are given as the means ±SE. Statistical significance is calculated according to Student's unpaired *t*-test.

MODIFICATIONS OF THE METHOD

Besides *Xenopus* oocytes (Waard and Campell 1995; Hering et al. 1997; Kraus et al. 1998), several other cell types and constructs, such as CHO cells (Sculptoreanu et al. 1993; Stephens et al. 1997), HEK293 (human embryonic kidney) cells (Lacinová et al. 1999), tsA-201 cells, a subclone of HEK293, (Peterson et al. 1997; McHugh et al. 2000), cardiac myocytes from rats (Scamps et al. 1990; Tohse et al. 1992; Gomez et al. 1994) and rabbits (Xu et al. 2000), isolated atrial myocytes from failing and non-failing human hearts (Cheng et al. 1996), skeletal muscle myotubes from mice and rabbits, (Johnson et al. 1994), myocytes of guinea pig mesentery artery (Morita et al. 1999), dendrites from rat pyramidal and olfactory bulb neurons (Markram and Sakmann 1994; Stuart and Spruston 1995; Koester and Sakmann 1998; Margie et al. 2001), rat amygdala neurons (Foehring and Srcoggs 1994; Young et al. 2001) were used to study the function of calcium channels.

Using the whole-cell variation of the patch-clamp technique, Yang et al. (2000) studied cellular T-type and L-type calcium channel currents in mouse neuroblastoma N1E115 cells. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 3–4 days. After mechanical agitation, 3×10^4 cells were replanted in 35-mm tissue culture dishes containing 4 ml of bath solution. After cell attachment, the dish was mounted on the stage of an inverted phase-contrast microscope for Ca^{2+} channel current recording. These cells expressed predominantly T channel currents. In experiments where L channels were specifically sought, the cells were grown and maintained at confluence for 3–4 weeks under the same culture condi-tions with the addition of 2% dimethylsulfoxide (Narahash et al. 1987). Three to 5 days before use, the

cells were replanted with the same medium. These cells expressed predominantly L channel currents. A small number of these cells also expressed T channel currents. Hence, cells were selected so that at a holding potential of –40 mV, the T channel component was very small and the inward current measured was conducted predominantly by L channels.

By using whole-cell and perforated patch-clamp techniques, Wu et al. (2000) showed that mifrabidile, a non-dihydropyridine compound, has an inhibitory effect on both T- and L-type Ca^{2+} currents in pancreatic β-cells.

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A.1.1.23.5 Patch clamp studies on chloride channels

PURPOSE AND RATIONALE

Cl^- channels are a large, ubiquitous and highly diverse group of ion channels involved in many physiological key processes including: regulation of electrical excitability; muscle contraction; secretion; and sensory signal transduction. Cl^- channels belong to several distinct families characterized in detail: voltage-gated Cl^- channels, the cAMP-regulated channel CFTR (cystic fibrosis transmembrane conductance regulator), ligand-gated Cl^- channels that open upon binding to the neurotransmitters GABA or glycine, and Cl^- channels that are regulated by the cytosolic Ca^{2+} concentration (Jentsch and Günther 1997; Frings et al. 2000).

Cliff and Frizel (1990) studied the cAMP- and Ca^{2+}-activated secretory Cl^- conductances in the Cl^- secreting colonic tumor epithelial cell line T84 using the whole-cell voltage-clamp technique.

PROCEDURE

T84 cells are used 1–3 days after plating on collagen-coating cover-slips. The cells are maintained at 37 °C. At this temperature, the responsiveness of the cells to secretagogues, particularly to cAMP-dependent agonists, is improved. Increases in Cl^- and K^+ conductances are the major electrical events during stimulation of Cl^- secretion. Accordingly, bath-pipette ion gradients are chosen so that transmembrane Cl^- and K^+ currents can be monitored independently at clamp voltages equal to the reversal potentials of these ions. The pipette solution is: 115 MM KCl, 25 mM *N*-methyl-D-glucamine (NMDG) glutamate, 0.5 mM EGTA, 0.19 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Na_2ATP, 0.05 mM Na_3GPT, 5 mM HEPES, pH 7.2. The bath solution is: 115 mM NaCl, 40 mM NMDG glutamate, 5 mM potassium glutamate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.2. Bath Na^+ and Cl^- concentrations are reduced by substituting NMDG chloride or sodium glutamate for NaCl. When Na^+ and K^+ free solutions are used, Na^+ and K^+ are replaced by $NMDG^+$, and Cl^- is reduced by replacing Cl^- by glutamate.

During whole-cell recording, the membrane potential is clamped alternately to three different voltages, each for 500-msec duration. Computer-controlled voltage-clamp protocols are used to generate current-voltage (*I-V*) relations when the transmembrane currents are relatively stable by stepping the clamp voltage between −100 and +100 mV at 20 mV intervals.

Test drugs (e.g., 8-(4-chlorophenylthio) adenosine 3',5'-cyclic monophosphate, A23187, forskolin, or ionomycin) are solubilized in stock solutions (ethanol of DMSO) and diluted.

EVALUATION

Instantaneous relations are constructed from currents recorded 6 msec after a voltage step.

MODIFICATIONS OF THE METHOD

Maertens et al. (2000) used the whole-cell patch-clamp technique to study the effect of an antimalarial drug on the volume-regulated anion channel (VRAC) in cultured bovine pulmonary artery endothelial cells. They also examined the effects on other Cl^- channels, i.e., the Ca^{2+} activated Cl^- channel and the cystic fibrosis transmembrane conductance regulator to assess the specificity for VRAC.

Pusch et al. (2000) characterized chloride channels belonging to the ClC family. Chiral clofibric acid derivates were tested on the human ClC-1 channel, a skeletal muscle chloride channel, after heterologous expression in *Xenopus laevis* oocytes by means of two microelectrode voltage clamp recordings.

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A.1.1.24 Measurement of cytosolic calcium with fluorescent indicators

PURPOSE AND RATIONALE

Intracellular free Ca-concentration can be measured in cultured endothelial cells with a fluorometric methods (Tsien et al. 1982; Grynkiewicz et al. 1985; Lückhoff et al. 1988; Busse and Lamontagne 1991; Hock et al. 1991).

PROCEDURE

Cultured endothelial cells from the pig are seeded on quartz coverslips and grown to confluence. The cells are loaded with the fluorescent probe indo-1 by incubation with 2 µmol indo-1/AM and 0.025% Pluronic® F-127, a non-ionic detergent. Thereafter, the coverslips are washed and transferred to cuvettes, filled with HEPES buffer.

EVALUATION

Fluorescence is recorded in a temperature controlled (37 °C) spectrofluorophotometer (excitating wavelength 350 nm, emission wavelength simultaneously measured at 400 and 450 nm).

MODIFICATIONS OF THE METHOD

Lee et al. (1987) measured cytosolic calcium transients from the beating rabbit heart using indo-1 AM as indicator.

Yangisawa et al. (1989) measured intracellular Ca^{2+} concentrations in coronary arterial smooth muscle of dogs with fura-2.

Makujina et al. (1995) measured intracellular calcium by fura-2 fluorescence simultaneously with tension in everted rings of porcine coronary artery denuded of endothelium.

Hayashi and Miyata (1994) described the properties of the commonly used fluorescent indicators for intracellular calcium: Fura-2, Indo-1, and Fluo-3.

Monteith et al. (1994) studied the Ca^{2+} pump-mediated efflux in vascular in spontaneously hypertensive rats.

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A.1.1.25 Measurement of contractile force of isolated cardiac myocytes

PURPOSE AND RATIONALE

Eschenhagen et al. (1997) developed a method for culturing embryonic cardiomyocytes in a collagen matrix to produce a coherently contracting 3-dimensional model heart tissue that allows direct measurement of isometric contractile force.

PROCEDURE

Ventricles from 9–11 day incubated chicken embryos (Cavanaugh 1955) are minced in Dulbecco's minimal essential medium (DMEM), washed once with 0.25% trypsin/0.1% EDTA in phosphate buffered saline (PBS), pH 7.45, and then digested in fresh trypsin/EDTA for 15 min at 37 °C. The supernatant is discarded and the pellet is subjected to digestion with 0.1% collagenase (144 U/mg) in PBS, pH 7.45, for 30 min at 37 °C. This supernatant is discarded and the pellet digested further with several cycles of collagenase for 10–20 min each until the pellet is completely digested. DNase I (40 µl, 1 mg/ml in PBS) is added between cycles depending on the presence of viscous DNA. The isolated cells are kept in Petri dishes in DMEM supplemented with 15% heat-inactivated fetal calf serum in the CO_2 incubator. After completion of the digestion, the cells are incubated for another 30–60 min in the CO_2 incubator (preplating). The cell suspension is centrifuged at 250 rpm (12 *g*). The pellet is resuspended in 10 ml culture medium (DMEM, 10% inactivated horse serum, 2% chicken embryo extract (Gibco BRL), 2 mmol/l glutamine, 10 µg/ml streptomycin, and 100 U/ml penicillin G, recentrifuged at 250 rpm, and finally resuspended in culture medium at $2–3 \times 10^6$ cells per ml.

For casting cardiomyocyte-populated collagen gels, strips of Velcro are glued with silicone rubber to glass tubes (13 mm length, 3 mm outer diameter, 2 mm inner diameter). Pairs of Velcro-coated tubes, kept at a fixed distance by a stainless steel wire spacer, are placed in rectangular wells ($15 \times 17 \times 4$ mm) cut into a layer of silicone rubber in a 100 mm polymethylenepentene Petri dish. This assembly is autoclaved before use. For each gel, 1 ml of an ice-cold collagen/cell mixture is poured into each well between the Velcro-coated glass tubes. This mixture has the same composition as the culture medium and contains in addition to 1 mg neutralized collagen I from rat tail (Upstate Biotechnol-

ogy, Inc.), 1×10^6 cardiomyocytes, the acetic acid in the collagen solution, and the NaOH to neutralize it. The mixture is allowed to gel at 37 °C for 60 min before culture medium is added to the dish. Medium changes are performed after overnight and then every other day.

After 6–11 days in culture, the gels are removed from the culture dish, the spacers are withdrawn, and one of the glass tubes is mounted on a fixed electrode; the other tube is connected by an inelastic silk string to an isometric force transducer attached to a Wekagraph thermal array recorder (Föhr Instruments, Heidelberg, Germany). The preparation is adjusted to its original (spacer) length before it is immersed in a convential water bath filled with modified Tyrode's solution maintained at 35 °C and continuously gassed with 95% O_2 and 5% CO_2.

After a 30–60 min equilibration period without pacing, force and frequency reaches a stable value. Gels are then electrically stimulated with rectangular pulses (10 ms, 20–40 V) at a standard frequency of 1.5 Hz. Preload is stepwise adjusted to L_{max}, the length at which the preparation develops maximal force. Cumulative doses of inotropic compounds, e.g. isoprenaline or forskoline, are added. All gels are exposed to a concentration-response curve for calcium (1.8–12.6 mmol/l) and one or two additional inotropic stimuli.

EVALUATION

All values are presented as arithmetic means ±SEM. Student's *t*-test for paired observations is used to compare force of contraction, resting tension, or beating frequency before and after other interventions.

MODIFICATIONS OF THE METHOD

Ferrara et al. (1997) studied the role of Gi-proteins and β-adrenoceptors in the age-related decline of contraction in guinea-pig ventricular myocytes. The isolated myocytes were placed in Krebs-Henseleit solution in a Perspex chamber on the stage of a Zeiss IM inverted microscope and superfused with Krebs-Henseleit solution containing 1 mmol/l Ca^{2+} at 2 ml/min and 32 °C. Cells were selected using the following criteria: rod shaped, without sarcolemmal blebs, no spontaneous contractions, stable baseline contraction to electrical stimulation at 0.5 Hz and sarcomere length not shorter than 1.67 µm. The image of the cells was displayed on a TV monitor and the length change measured with a video motion detector. Contraction amplitude and velocity was expressed as change in sarcomere length, calculated from the change in length of the myocyte and its original sarcomere length.

Using a similar technique, Harding et al. (1988) studied contractile responses of isolated rat and rabbit myocytes to isoproterenol and calcium, and Harding et al. (1992) isolated ventricular myocytes from failing and non-failing human heart.

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A.1.1.26 Adrenomedullin

A.1.1.26.1 General considerations

Adrenomedullin is a 52-amino acid peptide originally discovered in human adrenal pheochromocytoma by monitoring the elevating activity of platelet cAMP (Kitamura et al. 1993). Molecular cloning of rat adrenomedullin was reported by Sakata et al. (1993). The genomic structure of human adrenomedullin gene was reported by Ishimitsu et al. (1994). Adrenomedullin and **proadrenomedullin N-terminal 20 peptide (PAMP)** which are both hypotensive and bronchodilating, are derived from **preproadrenomedullin** (Kanazawa et al. 1995; Iwasaki et al. 1996; Shimosawa and Fujita 1996; Hinson et al. 1998; Samson 1998; Autelitano and Tang 1999; Jimenez et al. 1999; Lopez et al. 1999; Tajima et al. 1999).

Adrenomedullin is found ubiquitously in tissues and organs, especially in cardiovascular tissues and in the kidney, lung, brain and endocrine glands (Wimalawansa 1996; Van Rossum et al. 1997; Eto et al. 1999; Jougasaki and Burnett 2000; Kitamuro et al. 2000). The main biological effect is vasodilatation (Ishiyama et al. 1993). A hypotensive effect has been found in rats (Khan 1997), rabbits (Fukuhara et al. 1995), and man (Lainchbury et al. 1997). Adrenomedullin belongs to the calcitonin gene-related peptide/calcitonin peptide family as it shares approximately 25% homology with calcitonin gene-related peptide (Kitamura et al. 1993). Several pharmacological studies are related to the vasodilating

effect of adrenomedullin, e.g., in the mesenteric vascular bed (see A.8.2.5), (Santiago et al. 1995), in the hind limb vascular bed (see A.8.2.1), (Santiago et al. 1994; Champion et al. 1996, 1997), in the pulmonary vascular bed (see A.8.2.6), (DeWitt et al. 1994; Lippton et al. 1994; Heaton et al. 1995; Nossaman et al. 1995), on cerebral blood flow in dogs (Baskaya et al. 1995) and in cats (Takao et al. (1999), on renal hemodynamics in dogs (see A.8.2.3) (Ebara et al. 1994; Yukawa 1998), or on vasodilation in perfused rat kidneys (Hayakawa et al. 1999). Intravenous infusion of adrenomedullin exerted diuresis and natriuresis without major changes in blood pressure and produced beneficial hemodynamic and renal vasodilator effects in rats with compensated heart failure (Vari et al. 1996; Nagaya et al. 1999). In isolated perfused, paced rat heart preparations, adrenomedullin showed a dose-dependent inotropic effect (Szokodi et al. 1998). Pulmonary vasodilator responses and vasorelaxant effects in isolated pulmonary artery rings were found by Gumusel et al. (1998). Adrenomedullin is a growth-promoting factor for cultured vascular smooth muscle cells (Iwasaki et al. 1998) and fibroblasts (Isumi et al. 1998).

Willenbrock et al. (1999) showed a beneficial effect of adrenomedullin on renal function in rats with aortocaval shunt.

Adrenomedullin inhibits gastric secretion in rats with chronic gastric fistula (see J.3.1.3), (Rossowski et al. 1997) and inhibits reserpine-induced gastric lesions in rats (Clementi et al. 1998). Tsuchida et al. (1999) found an inhibition of cholecystokinin-stimulated amylase secretion by adrenomedullin in rat pancreatic acini.

Lewis et al. (1998) described a specific and sensitive radioimmunoassay for human adrenomedullin.

Ohta et al. (1999) developed an one-step direct assay for adrenomedullin with monoclonal antibodies.

N-terminal fragments of adrenomedullin show vasopressor activities (Watanabe et al. 1996).

Adrenotensin, an other adrenomedullin gene product, contract in an endothelium-dependent manner pulmonary blood vessels (Gumusel et al. 1996).

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A.1.1.26.2 Receptor binding of adrenomedullin

PURPOSE AND RATIONALE

Muff et al. (1995), Poyer (1997) reviewed the binding characteristics of the structurally related hormones calcitonin, calcitonin gene-related peptide, amylin, and adrenomedullin. Vine et al. (1996) compared *in vitro* binding of adrenomedullin, calcitonin gene-related peptide and amylin.

Specific adrenomedullin binding sites were described in human brain (Sone et al. 1997), in the rat spinal cord (Owji et al. 1996), in cultured brain cells (Zimmermann et al. 1996), and in cultured rat mesangial cells (Osajima et al. 1996).

PROCEDURE

Human brain is obtained at autopsy. For preparation of membranes, tissues are homogenized in ice-cold 50 mM HEPES buffer, pH 7.6; containing 0.25 M sucrose, 10 µg/ml soybean trypsin inhibitor, 0.5 µg/ml pepstatin, 0.5 µg/ml leupeptin, 0.5 µg/ml antipain, 0.1 mg/ml benzamidine, 0.1 mg/ml bacitracin, and 30 µg/ml aprotinin. The homogenates are centrifuged at 1 500 *g* for 20 min at 4 °C. The pellets are resuspended in 10 vol of the above buffer without sucrose and centrifuged at 100 000 *g* for 1 h at 4 °C. The final pellets are resuspended to a concentration of 2–10 mg protein/ml, aliquoted, and stored at –80 °C.

For the receptor binding assay, brain membranes (100 µg protein) are incubated at 4 °C in 0.5 ml binding buffer (20 mM HEPES buffer, pH 7.4, containing 5 mM $MgCl_2$, 10 mM NaCl, 4 mM KCl, 1 mM EDTA, and 0.3% BSA) containing 0.3 nM ^{125}I- human adrenomedullin in siliconized microcentrifuge tubes. Pellets are washed with 0.5 ml binding buffer at 4 °C and counted in a γ-counter.

EVALUATION

Nonspecific binding is determined in the presence of 200 nM unlabeled human adrenomedullin. Specific binding is defined as total binding minus nonspecific binding. Data are calculated as mean ±SEM.

MODIFICATIONS OF THE METHOD

Eguchi et al. (1994) studied the binding of human adrenomedullin and analogs and the adenylate cyclase activity in cultured rat vascular smooth muscle cells.

Moody et al. (1997) investigated the binding affinity of adrenomedullin in C6 glioma cells.

Findings of Belloni et al. (1998) suggested the existence of different receptor subtypes for adrenomedullin in the human adrenal cortex.

Mazzocchi et al. (1999) found abundant [^{125}I]-adrenomedullin binding sites in both zona glomerulosa and adrenal medulla in the rat adrenal gland.

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A.1.1.27 Atrial natriuretic factor (ANF)

A.1.1.27.1 General considerations

PURPOSE AND RATIONALE

The atria of mammalian hearts synthesize and secrete peptides with potent natriuretic and vasoactive properties known as ANF = atrial natriuretic factor (de Bold et al. 1981). The atrial natriuretic peptide hormonal system consists of a 126-amino acids prohormone synthesized within myocytes of the heart and stored in storage granules within the heart before release into circulation (Kangawa and Matsuo 1984; Oikawa et al. 1984; Vesely 1992). This hormonal system contains several peptides from the 126-amino acid-prohormone with blood pressure lowering, natriuretic, diuretic and/or kaliuretic properties (Martin et al. 1990; Vesely et al. 1994). Thus, peptides consisting of amino acid 1 to 30 (LANP = long-acting natriuretic peptide), 31 to 67 (vessel dilator), 79 to 98 (kaliuretic peptide) and 99 to 126 (ANF) each have blood pressure lowering, natriuretic, diuretic and/or kaliuretic properties both in humans and

in animals. Human and rat atria predominantly secrete a peptide of 28 amino acid residues, ANF-(99-126), which represents the C-terminus of a precursor sequence of 126 amino acid residues. In addition, vessel dilator and LANP circulate as distinct entities after having been proteolytically cleaved from the rest of the amino terminus by proteases (Ackerman et al. 1997).

Plasma immunoreactive ANF-(99-126) concentration increases in normal rats after volume expansion, while infusion of the peptide lowers blood pressure in several animal models of hypertension.

An international standard for atrial natriuretic factor was established by an international collaborative study (Poole et al. 1988). Human ANF-(99-126) was synthesized, highly purified and distributed to several laboratories, who performed radioimmunoassays, radioreceptor assays and an *in vitro* assay using the vasorelaxant activity in precontracted rat aortic strips.

The C-type natriuretic peptide is a 22-amino acid peptide that was initially identified in the central nervous system (Ogawa et al. 1992; Barr et al. 1996; Amin et al. 1996). The distribution of C-type natriuretic peptide, which has structural homology with atrial and brain natriuretic peptides and also similar activities, is wide and includes the endothelium, myocardium, gastrointestinal and genito-urinary tracts.

Jiao and Baertschi (1993) reviewed the neural control of the endocrine rat heart. Stimulation of cardiac sympathetic nerves potently stimulates ANF secretion.

ANF inhibits proliferation in non-myocardial cells and is anti-hypertrophic in cardiomyocytes. Silberbach et al. (1999) reported that activation of an extracellular signal-regulated protein kinase is required for the anti-hypertrophic effect of atrial natriuretic factor in neonatal rat ventricular myocytes.

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A.1.1.27.2 Bioassay for ANF

PURPOSE AND RATIONALE

Matsui et al. (1987) described a rapid bioassay for quantification of atrial natriuretic polypeptides in rats with continuous recording of the conductivity of the urine, urine flow and blood pressure.

PROCEDURE

Male Sprague Dawley rats weighing 180–240 g are anesthetized with 60 mg/kg i.p. pentobarbital sodium. Anesthesia is maintained by injection of supplemental doses of pentobarbital sodium. After tracheotomy, catheters are placed into the left jugular vein and the right carotid artery for injection of samples or infusion of 10% mannitol in 0.9% saline and for blood pressure recording. Through a small suprapubic incision the bladder is cannulated for collection of urine, and the cannula is connected to a device for continuous measurement of urine conductivity, by which the electrolyte concentration is estimated. Urine flow rate is recorded using a drop counter, and urine samples are collected in a plastic tube.

After completion of surgery, 0.6–0.8 ml of 10% mannitol in 0.9% saline is administered into the jugular vein and is continuously infused at a rate of 4.0 ml/h by a syringe pump. Following an equilibration of 45–60 min, the bioassay is started when the urine flow is increased to 50–75 µl/min. All test samples with a volume of 100 µl are directly injected into the jugular vein followed by a wash injection of ~30 µl of saline. Mean arterial blood pressure, urine conductivity, and urine flow rate are simultaneously recorded.

For dose-response curves, serial dilutions of human ANF (α-hANP) and of test substance are prepared. Vehicle and various doses of α-hANP or test substance are injected in a randomized sequence. Immediately after the injection, urine is collected for 10 min. Urine volumes are determined by weighing, and urinary sodium and potassium concentrations are measured by flame photometry.

EVALUATION

Linear-regression analyses by the method of least squares are used for evaluating dose-response relationship. One-way analysis of variance for repeated measures and the Newman-Keuls test are used to detect statistical differences.

MODIFICATIONS OF THE METHOD

Petersen et al. (1988) determined atrial content and plasma levels of atrial natriuretic peptides in rats with chronic renal failure. The natriuretic activity in the bioassay was estimated as the increase in Na excretion in urine samples from the control period to the maximal natriuretic response.

Allen and Gellai (1987) measured cardio-inhibitory effects of atrial peptide in conscious chronically instrumented rats. The hemodynamic and renal excretory responses were measured with and without replacement of urinary fluid losses.

Thibault et al. (1984) characterized the biological activities of atrial natriuretic factor-related peptides *in vivo* by a natriuretic bioassay and *in vitro* by relaxation of contracted intestinal smooth muscle (chick rectum).

Schiller et al. (1986) tested synthetic analogs of atrial natriuretic peptide in the rabbit aorta assay and in a bioassay monitoring suppression of aldosterone secretion from bovine zona glomerulosa cells.

Dlouha and McBroom (1986) measured diuretic and natriuretic activity of atrial extracts of taurine-treated normal and cardiomyopathic hamsters by urine flow and Na^+ excretion in the rat bioassay.

Kohse et al. (1992) described a bioassay for quantitative determination of natriuretic peptides in human biological samples using bovine aortic and bovine kidney epithelial cultured cells. The amount of cyclic AMP produced by these cells was measured by radioimmunoassay.

Kecskemeti et al. (1996) studied the effects of atrial natriuretic peptide (ANP) on action potential characteristics in various (human, rabbit, guinea-pig) atrial and guinea pig ventricular papillary muscles. The data suggested that ANP inhibits the slow inward Ca^{2+} channel activity and facilitates the K^+ channel activity.

Salt-sensitive hypertension was found in ANP knockout mice (Melo et al. 1998).

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A.1.1.27.3 Receptor binding of ANF

PURPOSE AND RATIONALE

Schiffrin et al. (1985) described receptors for atrial natriuretic factor in the rat.

PROCEDURE

Synthetic ANF-(99-126) is iodinated with ^{125}I by a modification (Gutskowska et al. 1984) of the chloramine T method (Greenwood and Hunter 1963). Separation of radiolabeled ANF from free iodine is achieved by immuno-affinity chromatography followed by C-18 reverse phase high pressure liquid chromatography.

For preparation of membranes, Sprague Dawley rats weighing 300 g are sacrificed by decapitation. The atria, ventricles, renal arteries, mesentery, the mesentery vascular bed, and adrenals are processed for binding studies. Adrenal capsules are separated by manual compression. The tissues are immersed in 0.25 M sucrose solution, finely minced with scissors, and homogenized in a Polytron (setting 8, 10 s twice). The

homogenate is centrifuged at 1 550 *g* for 10 min at 4 °C; the supernatant is decanted and recentrifuged. The final supernatant is filtered through a cheesecloth, then centrifuged at 10 400 *g* for 30 min. The pellet is resuspended in a 0.05 M Tris-HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM $MgCl_2$, 0.5 mM phenyl methyl sulfonyl fluoride, 0.1% bacitracin, and 1 µM aprotinin. Proteins are measured by the Coomassie blue method (Spector 1978). Next, bovine serum is added at a concentration of 0.2%, and the membranes are diluted to a protein concentration of 0.25 to 1 mg/ml in the Tris-buffer containing 0.2% albumin.

The ^{125}I-AFN binding assay uses 30–50 pM of labeled ANF and 10^{-13} to 10^{-6} unlabeled ANF in competition experiments. In saturation experiments, increasing concentrations of ^{125}I-AFN (6–200 pM) are used, and nonspecific binding is determined by incubation in the presence of 1 µM unlabeled ANF for each point of the saturation curves. Incubation is done with 25–100 µg of receptor protein per tube, at 4 °C for 60 min. All assays are performed in duplicate. Separation of bound and free radioactivity is achieved by rapid filtration through polyethylenimine-treated Whatman GF/C filters soaked with the assay buffer. The filters are washed twice with 3 ml of 0.9% NaCl, then are allowed to dry and are counted in a gamma counter.

EVALUATION

Binding data are analyzed by computer assisted nonlinear regression analysis using the LIGAND program (Munson and Rodbard 1980). The inhibition constant K_i is calculated according the Cheng and Prussoff equation.

MODIFICATIONS OF THE METHOD

Misono (2000) found that the binding of atrial natriuretic factor to its receptor is dependent on chloride concentration.

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A.1.1.27.4 ANF gene expression

PURPOSE AND RATIONALE

Production of atrial natriuretic factor and brain natriuretic peptide can be measured by gene expression using total RNA extraction and Northern blot analysis and the quantitative competitive reverse transcription polymerase chain reaction (Hama et al. 1995; Ogawa et al. 1996, 1997, 1998, 1999).

PROCEDURE

Extraction of plasma and tissue samples

Plasma samples are acidified by adding 100 µl/ml of 1 mol/l HCl and passed through Sep-Pak C_{18} cartridges (Millipore) that are prewetted with 5 ml of 80% acetonitrile in 0.1% trifluoroacetic acid (TFA) and 10 ml of 0.1% TFA. The cartridges with the absorbed peptides are washed with 20 ml of 0.1% TFA and eluted with 3 ml of 60% acetonitrile in 0.1% TFA. Tissue samples are homogenized in 10 vol of an extracting mixture consisting of 0.1 N HCl, 1.0 mol/l acetic acid, and 1% NaCl and centrifuged at 10 000 *g* for 30 min at 4 °C. The supernatants are then extracted with the use of Sep-Pak C_{18} cartridges by elution with 80% acetonitrile in 0.1% TFA. The eluates from tissue or plasma are freeze-dried and processed for RIA.

Total RNA extraction and northern blot analysis

Atrial and ventricular tissue samples from individual rats are extracted using Trizol (GIBCO BRL). Total RNA from the atrium (10 µg) and ventricle (20 µg) are electrophoretically separated in an agarose-formaldehyde gel followed by blotting to nylon membranes (Hybond N+, Amersham) overnight. Membranes are prehybridized in 2.5× Denhardt's solution, 5× SSC, 50% formamide, 25 mmol/l KH_2PO_4, pH 6.4, 0.2% SDS, and 0.2 mg/mL herring sssDNA for 3 h at 42 °C for cDNA probes, or prehybridized in 5× Denhardt's solution, 6× SSC, 50 mmol/l NaH_2PO_4, 0.5% SDS, and 0.2 mg/ml herring sssDNA for 3 h at 5 °C below the calculated T_m for oligonucleotide probes. Hybridization is then carried out for 16 h at the same temperature and the same solution as the prehybridization condition except for the presence of the radiolabeled probes. Five cDNA probes and two oligonucleotide probes are used. The cDNA probes used are as follows: (1) a 900-bp *Eco*RI/*Hin*dIII fragment containing the full-length rat ANF cDNA, (2) a 595-bp *Sal*I fragment containing full-length rat BNP cDNA, (3) a 5-kb *Eco*RI/*Sal*I fragment of the mouse 28S rRNA cDNA probe, (4) a 2-kb *Bam*HI/*Bgl*II fragment of the mouse PGK gene cDNA, and (5) rat α_1-III collagen

cDNA containing 1 300 bp of the 3' noncoding and coding regions. The two oligonucleotide probes are 39 and 24 base fragments specific for unique regions in the 3' untranslated regions of the rat α-MHC and β-MHC genes. The α-sequence is

5'GGGATAGCAACAGCGAGGCTCTTTCTGC-TGGACAGGTTA-3' (T_m = 60°C),

and the β-sequence is

5'-CTCCAGGTCTCAGGGCTTCACAGG-3' (T_m = 52°C).

The cDNAs are labeled with 5'-[α^{32}P]dCTP (3 000 Ci/mmol, Amersham) using the Megaprime DNA labeling system (Amersham). The oligonucleotides are labeled with [γ-^{32}P]ATP (3 000 Ci/mmol, Amersham) using a 5'-end-labeling kit (Amersham). At the end of hybridization, the membranes are washed twice at 42 °C with 2× SSC and 1% SDS and twice at 55 °C with 1× SSC and 0.1% SDS for the cDNA probes or are washed once at 30 °C with 5× SSC and 0.1% SDS and twice at the same temperature as the hybridization with 1× SSC and 0.1% SDS for the oligonucleotide probes. Before additional probing, bound counts are completely stripped from the membranes by washing twice in 10 mmol/l sodium citrate, pH 6.8, 0.25% SDS for 10 min at 100 °C. Autoradiographs are scanned with an Ultrascan XL laser densitometer (LKB Produckter) and LKB 2400 Gelscan XL software package. The scanning values of ANF, BNP, collagen-III, and α-MHC and β-MHC mRNAs are normalized to 28S ribosomal RNA or PGK mRNA as internal controls to correct for differences in the amount of RNA applied and transfer efficiency.

Plasma and cardiac tissue concentrations of immunoreactive ANF and BNP are determined by RIA with anti-rat $ANF_{99–126}$ and anti-rat $BNP_{64–95}$ sera, respectively, from Peninsula Laboratories.

Quantitative competitive reverse transcription polymerase chain reaction

RNA samples are reverse transcribed with Super Script II RNase H 2 Reverse Transcriptase and oligo(dT)12–18 primer with the use of a reverse transcription kit (GIBCO BRL). An aliquot of the cDNA product is used for PCR amplification with ANF primers. A dilution series of total RNA (5 mg) aliquots is prepared for each sample. Each dilution is spiked with competitor RNA. After the PCR, aliquots (5 ml) of the PCR product are electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. Photographs are taken with Polaroid 55 film, and the negatives are scanned with the use of an Ultrascan XL laser densitometer and Gelscan XL 2000 software package. The ratio of the density of the competitor RNA to the target RNA is plotted against the amount of the competitor RNA added to each reaction.

EVALUATION

All results are expressed as mean ±SEM. A level of $P < 0.5$ is considered significant. ANOVA is performed to determine statistical differences among multiple groups. When significance is obtained by ANOVA, Fisher's least squares difference post hoc analysis is used to determine pairwise differences.

MODIFICATIONS OF THE METHOD

Ramirez et al. (1997) reported that the nuclear δ_B isoform of Ca^{2+}/calmodulin-dependent protein kinase II regulates atrial natriuretic gene expression in cultured neonatal rat ventricular myocytes.

Thuerauf et al. (1998) found that the p38 mitogen-activated protein kinase mediates the transcriptional induction of the atrial natriuretic factor gene through a serum response element and discussed the potential role for the transcription factor ATF6.

Kakita et al. (1999) studied p300 protein as a co-activator of the transcription factor GATA-5 in the transcription of cardiac-restricted atrial natriuretic factor gene.

Bianciotti and de Bold (2000) investigated the effect of selective ET_A receptor blockade on natriuretic peptide gene expression in DOCA-salt hypertension in rats.

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A.1.1.27.5 Radioimmunoassay for ANF

PURPOSE AND RATIONALE

Gutkowska et al. (1984) developed a direct radioimmunoassay of atrial natriuretic factor (ANF). The method uses a synthetic 26-amino-acid fragment (8–33 ANF) of the native peptide.

PROCEDURE

Because 8–33 ANF is a small molecule, it is necessary to covalently conjugate the peptide to a larger protein (bovine thyroglobulin) for immunization. To 50 mg thyroglobulin dissolved in 2 ml distilled water, pH 7.4, 30 mg CDI [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl] is added in 1 ml distilled water, pH 7.4. Then 5 mg 8–33 ANF in water is added dropwise while stirring. The solution is kept overnight at 4 °C, then another 30 mg CDI is added and the mixture is kept for 2 h at room temperature with constant stirring. The cloudy mixture is dialyzed for 24 h at 4 °C against 0.9% saline. The dialyzed material is then fractionated and stored at –70 °C.

For immunization, 100 µg of the ANF-thyroglobulin complex are suspended in 1 ml saline, thoroughly mixed with 1 ml complete Freund's adjuvant and injected into the shaved backs of New Zealand white rabbits. Each animal receives also 0.5 ml *Bordetella pertussis* vaccine subcutaneously with the primary immunization. The animals are re-immunized at monthly intervals with 100 µg of antigen in incomplete Freund's adjuvant and bled by ear artery 10 days after the booster injection.

For iodination, 5 µg ANF in 5 µl 0.01 M ammonium acetate, pH 5.0 is introduced in a 1.5 ml Eppendorf vial followed by the addition of 1 mCi Na ^{125}I in a volume of 25 µl. Chloramine T 10 µg/10 µl is added to the reaction vial and 30 s later sodium metabisulfide (20 µg/10 µl) is added. Each addition is followed by mixing. Purification of the iodinated tracer is achieved by HPLC on a µBondapax C_{18} column, eluted with a linear gradient of 20 to 50% acetonitrile with 0.1% trifluoroacetic acid with a slope of 0.5%/min and a flow rate of 1 ml/min.

The radioimmunoassay procedure is performed in polystyrene tubes at 4 °C by mixing 100 µl of standard or sample, 100 µl of antiserum diluted 1:4 000, 100 µl of ^{125}I-ANF and 300 µl of the same buffer containing 1% BSA. After incubation for 24 h at 4 °C the free from antigen-bound ^{125}I-ANF is separated by dextran-coated charcoal. One ml of dextran-charcoal suspension is added to each tube. After 5 s agitation the tubes are centrifuged at 4 000 rpm at 4 °C for 10 min. The supernatant is decanted and the radioactivity counted in a gamma counter.

EVALUATION

Dose-response curves are prepared and Scatchard analysis is performed.

MODIFICATIONS OF THE METHOD

Radioimmunoassays were also developed for long-acting natriuretic peptide and vessel dilator (Vesely et al. 1994; Winters et al. 1989).

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A.1.2 Studies in isolated organs

A.1.2.1 α-Sympatholytic activity in isolated vascular smooth muscle

PURPOSE AND RATIONALE

Noradrenaline and other sympathomimetic drugs increase vascular smooth muscle tone by stimulation of α-adrenergic receptors. Contractions can be antagonized by α-adrenergic receptor blocking agents such as phentolamine. Drugs can be tested for their capacity of reducing vascular smooth muscle contractions induced by the adrenergic receptor-activating agent noradrenaline. Moreover, effects of peptides, such as bradykinin, can be tested with strips of aorta or pulmonary artery.

PROCEDURE

As donor animals Pirbright White guinea pigs of either sex weighing about 400 g, or Chinchilla rabbits weighing about 3.5 kg, or Sprague-Dawley rats weighing 200–300 g are used. The vessels to be tested are the thoracic aorta or the arteria pulmonalis. The animals are sacrificed by stunning and exsanguination. The pulmonary artery or the thoracic aorta is quickly removed and cut into helical strips of 1–2 mm width and 15–20 mm length. The strips are mounted in an organ bath with a preload of 1 g. Krebs-Henseleit buffer solution containing 11.5 M glucose is maintained at 37 °C and oxygenated with 95% O_2, 5% CO_2. Isotonic or isometric registration is performed. Changes in length are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronik Freiburg). Isometric force is measured with a force transducer (UC-2 Gould-Statham, Oxnard, USA).

Experimental course

Following an equilibration period of 60 min, contractions are induced by repeated administrations of (–)noradrenaline HCl in concentrations of 2×10^{-6} M for testing the contractions of the pulmonary artery and in concentrations of 2×10^{-8} M for testing the contractions of the aorta. After obtaining a stable plateau of identically sized contractions, cumulative doses of the test compound are added into the organ bath. Consecutive concentrations are given when the response of the previous dose has reached a plateau.

Controls at the end of the experiment: If a compound does not show vasorelaxing activity at any dose, the sensitivity of the preparation is tested by adding phentolamine (1×10^{-7} M).

If a compound shows vasorelaxing activity, the reversibility of the relaxation is tested by increasing the noradrenaline concentration.

EVALUATION

The contractile force is determined before and after drug administration.

Percent inhibition of spasmogen-induced contraction by test drug is calculated as compared to the maximal contraction with a spasmogen alone (= 100%).

IC_{50} values are determined from the individual dose-response curves. IC_{50} is defined as the dose of drug leading to a 50% relaxation of noradrenaline-induced contraction.

MODIFICATIONS OF THE METHOD

The isolated vena cava of rabbits can be used for assaying α-adrenolytic activity. The rabbit is sacrificed by CO_2 anesthesia. The vena cava inferior is removed and cut into strips. The percent inhibition of epinephrine or norepinephrine induced contractions is determined.

The effects of bradykinin and bradykinin antagonists can be tested in isolated guinea pig artery and isolated rabbit aorta which contains predominantly the BK_1-receptor type (Regoli and Barabé 1980; Hock et al. 1991).

Wisskirchen et al. (1998) tested agonists of calcitonin gene-related peptide, homologues and antagonists in rat isolated pulmonary artery. Endothelium-intact pulmonary artery rings were contracted with 3×10^{-8} M phenylephrine and a cumulative dose-response curve of relaxation was constructed.

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A.1.2.2 α-Sympatholytic activity in the isolated guinea pig seminal vesicle

PURPOSE AND RATIONALE

The seminal vesicles of guinea pigs and rats are tubular organs whose longitudinal and annular muscles are innervated by the sympathetic system. The inhibition of contractions induced by norepinephrine or the α_1-selective agonist phenylephrine indicates α-sympatholytic activity. Sharif and Gokhale (1986) recommended the use the isolated rat seminal vesicle as a rather sensitive and specific model.

PROCEDURE

Male guinea pigs weighing 300 to 600 g are sacrificed by a blow to the neck. Both seminal vesicles are prepared and placed in Ringer's solution in an organ bath

maintained at 32 °C and being oxygenated with 95% O_2/5% CO_2. Isotonic or isometric registration is performed. Changes in length are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronik Freiburg). Isometric force is measured with a force transducer (UC-2 Gould-Statham, Oxnard, USA).

Following an equilibration period of 30 min, contractions are induced by repeated administration of (–)norepinephrine HCl in concentrations of 1 to 5 µg/ml or phenylephrine HCl in concentrations of 10 to 50 µg/ml. After obtaining a stable plateau of identical contractions, the test compound is added into the organ bath. Three min later, the previous concentration of norepinephrine or phenylephrine is added. As standard phentolamine is used in concentrations of 3 to 30×10^{-7} M.

EVALUATION

Contractions of the seminal vesicle induced by the α-adrenergic agonists after addition of the test compound are compared with the initial values and expressed as percentage thereof. For in depth analysis, full dose-response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x – values according to Schild (1947)

MODIFICATION OF THE METHOD

Leitch (1954) recommended the use of isolated seminal vesicles of rats for the assay of sympatholytic drugs.

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A.1.2.3 α-Sympatholytic activity in the isolated vas deferens of the rat

PURPOSE AND RATIONALE

The vas deferens of the guinea pig or preferably the rat is used for quantitative evaluation of adrenergic antagonists. The response of this organ to α-adrenergic agonists consists of a strong rapid contraction followed by quick relaxation on washing the agonists out of the tissue.

PROCEDURE

Male Wistar rats weighing about 300 g are used. The animals are sacrificed by a sharp blow to the neck, the vasa deferentia are dissected free from the extraneous tissues and suspended in a organ bath containing Tyrode solution being oxygenated with a 95% O_2 and 5% CO_2 mixture at 32 °C. Isotonic registration is performed at a preload of 0.5 g. Changes in length are recorded isotonically using a lever transducer (e.g., 368 type B, Hugo Sachs Elektronik, Freiburg, FRG).

Following an equilibration period of 30 min, contractions are induced by repeated administration of (–)norepinephrine HCl in concentrations of 0.5, 1.0, 2.0, or 4.0 µg/ml. After obtaining a stable plateau of identical contractions, the test compound is added into the organ bath. Three min later, the previous concentration of norepinephrine is added. As standard phentolamine is used in concentrations of 3 to 30×10^{-7} M.

EVALUATION

Contractions of the vas deferens induced by the α-adrenergic agonist after addition of the test compound are compared with the initial values and expressed as percentage thereof. For in depth analysis, full dose-response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x – values according to Schild (1947).

MODIFICATIONS OF THE METHOD

Electrical stimulation of the isolated ductus deferens results in the release of norepinephrine. Stimulation induced contractions of this organ are inhibited by clonidine which impairs adrenergic neurotransmission by activating inhibitory α-receptors. The ductus deferens is suspended in an organ bath bubbled with carbogen and maintained at 37 °C. Tension is adjusted to 25 mN. Following a 45 min equilibration period, supramaximal amplitude stimulation by a HSE type 2 stimulator (Hugo Sachs Elektronik, Freiburg) is applied. After stabilization of the response, clonidine is added to the organ bath in accumulated doses. Test compounds are added 5 min prior to clonidine administration. The percent potentiation of clonidine induced inhibition of contractions is determined.

Taylor et al. (1983) used the rat deferens for pharmacological characterization of purinergic receptors.

Nerve-muscle preparations of the vas deferens have been reviewed by Holman (1975).

Hughes et al. (1974) used the electrically stimulated mouse vas deferens for assessment of the agonistic and antagonistic activities of narcotic analgesic drugs.

Ross et al. (2001) used the mouse vas deferens to study structure-activity relationship for the endogenous cannabinoid, anadamide, and certain of its analogues at vanilloid receptors.

Oka et al. (1980) recommended the vas deferens from rabbits as a specific bioassay for opioid κ-receptor agonists.

Mutafova-Yambolieva and Radmirov (1993) studied the effects of endothelin-1 on electrically- or drug-induced contractile responses mediated by purinergic or adrenergic receptors in the isolated prostatic portion of rat vas deferens.

Ward et al. (1990) used isolated vasa deferentia preparations from rat and mouse to study the pharmacological profile of the analgesic pravadoline.

Hukovic (1961) described an isolated ductus deferens preparation together with the sympathetic hypogastric nerve of the guinea pig.

Cordellini and Sannomiya (1984) pretreated guinea pigs with reserpine. In the isolated vasa deferentia concentration-effects curves to phenylephrine were established in the presence of cocaine. The antagonistic effect of phenoxybenzamine was used for receptor occupancy studies.

Donoso et al. (1992) studied neurotransmission in epididymal and prostatic segments of isolated superfused rat vas deferens preparations.

Vaupel and Su (1987) used the vas deferens preparation of guinea pigs to study sigma and phencyclidine receptors.

Eltze (1988) used the field-stimulated (95% of maximum voltage, 0.1 Hz, 0.5 ms) portion of rabbit vas deferens to study muscarinic M_1 and M_2-receptors.

Dumont et al. (1997) used the isolated guinea pig heart and the isolated rat vas deferens for *in vitro* bioassays of calcitonin gene-related peptide (CGRP) agonists and antagonists.

Poyner et al. (1999) found concentration-dependent inhibitions of the electrically stimulated twitch responses of guinea pig vas deferens by calcitonin gene-related peptide, amylin and adrenomedullin.

Wisskirchen et al. (1998) tested agonists of calcitonin gene-related peptide, homologues and antagonists in rat isolated vas deferens. The prostatic half was suspended under 0.5 g resting tension and equilibrated in Krebs solution at 37 °C. Contractile responses of the prostatic vas were induced by electrical field stimulation at 0.2 Hz, 1.0 ms and 60 V through parallel platinum electrodes either side of the tissue.

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A.1.2.4 α-Sympatholytic activity in the isolated rat spleen

PURPOSE AND RATIONALE

α-Stimulant agents (such as epinephrine, norepinephrine) or electrical stimulation induce contractions in sympathetically innervated organs such as spleen smooth muscle. These effects can be antagonized by drugs with α-blocking activities such as phentolamine.

PROCEDURE

Male Sprague-Dawley rats weighing 180–220 g are used. The animal is sacrificed in CO_2 anesthesia. The spleen is removed and cut longitudinally into two halves. Each part is placed in an organ bath containing nutritive solution. The bath solution is bubbled with carbogen and maintained at 37 °C. Following a 30 min incubation period under a tension of 0.5 g, contractions are elicited by administration of epinephrine (10^{-6} g/ml) or norepinephrine 10^{-6} g/ml). After obtaining 3 approximately identical spasms, the test compound is administered followed by the addition of the spasmogen 5 min later. The contractile response is allowed to plateau and recorded.

Standard compound:

- phentolamine

EVALUATION

The contractile force is recorded at its maximal level before and after drug administration. The percent inhibition of epinephrine or norepinephrine induced contraction is determined.

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A.1.2.5 α-Sympatholytic activity in the isolated rat anococcygeus muscle

PURPOSE AND RATIONALE

The rat anococcygeus muscle as pharmacological tool was introduced by Gillespie (1972, 1980), Gibson and Gillespie (1973). This smooth muscle has a dense adrenergic innervation and contracts to noradrenaline, acetylcholine, 5-hydroxytryptamine, but not to histamine. Moreover, the muscle contracts to field stimulation or stimulation of extrinsic nerves. The preparation can be used to assess the pre- and post-synaptic α-adrenoceptor blocking activity of drugs (Doggrell 1980, 1983).

PROCEDURE

The two anococcygeus muscles arise from the upper coccygeal vertebra close to one another in the midline of the pelvic cavity. The muscles pass caudally, lying first behind and the to one side of the colon, finally joining together to form a ventral bar in front of the colon a few mm from the anus. The extrinsic nerves pass in a branch of the perineal nerve on either side to enter the deep surface of each muscle just before the formation of the ventral bar.

After sacrifice, the abdomen of rats is opened in the mid-line, the pelvis split and the bladder and urethra removed. Care is required in clearing the lower part of the urethra to avoid damage to the ventral bar of muscle, the only region lying ventral to the colon. The colon is then cut through at the pelvic brim, the pelvic portion pulled forward and the delicate connective tissue behind cleared until the anococcygeus muscles come into view. The muscles are isolated, in some instances with the extrinsic nerve intact. The extrinsic nerves on either side run in the posterior scrotal branch of the perineal nerve and leave it to enter the deep surface of the anococcygeal muscles as the lie on the lateral surface of the colon. The ventral bar is cut through and each muscle mounted in a 100 ml bath containing Krebs solution at 36 °C. The solution is gassed with 95% O_2 + 5% CO_2. Tension is measured with isometric transducers and displayed on a polygraph. Field stimulation of the intramural nerves is applied after drawing the muscles through a pair of electrodes similar to those described by Burn and Rand (1960); when the muscles are stimulated through their extrinsic nerves the nerves are drawn through similar electrodes. Stimulation of either intramural or extrinsic nerves is with 1 ms pulses at 20 Hz, and at a supramaximal voltage.

Dose-response curves are established with doses of 2×10^{-7} to 4×10^{-6} M noradrenaline, 4×10^{-7} to 4×10^{-5} M acetylcholine or with graded frequencies of electrical stimulation.

The effect of noradrenaline is abolished by α-adrenergic antagonists, such as 10^{-6} M phentolamine. Dose-response curves show a parallel shift characteristic of competitive antagonism.

EVALUATION

Contractions of the anococcygeus muscle induced by an α-adrenergic agonist after addition of the test compound are compared with the initial values and expressed as percentage thereof. For in depth analysis,

full dose-response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x – values according to Schild (1947).

MODIFICATIONS OF THE METHOD

Gibson et al. (1990) found L-N^G-nitroarginine to be a potent inhibitor of non-adrenergic, non-cholinergic relaxations in the rat anococcygeus muscle.

Oliveira and Bendhack (1992) found that dopamine has a dual effect in the rat anococcygeus muscle: a partial effect due to an indirect sympathomimetic action and a partial effect due the interaction with postjunctional receptors.

Brave et al. (1993) investigated the interaction between motor sympathetic and inhibitory non-adrenergic, non-cholinergic nerves in the rat anococcygeus muscle using L-N^G-nitro-arginine, an inhibitor of L-arginine:NO synthase.

Cakici et al. (1993) described a co-axial bioassay system consisting of guinea pig trachea as donor organ for epithelial derived-relaxing factors and phenylepinephrine-precontracted rat anococcygeus muscle as assay tissue.

Iravani and Zar (1993) found differential effects of nifedipine on nerve-mediated and noradrenaline-evoked contractions of rat anococcygeus muscle.

Rand and Li (1993) studied the modulation of acetylcholine-induced contractions of the rat anococcygeus muscle by activation of nitrergic nerves.

Mudumbi and Leighton (1994) investigated the mechanisms of action of relaxation induced by bradykinin and by electrical field stimulation in isolated rat anococcygeus muscle, where contractile tone had been elevated with clonidine.

Gwee et al. (1995) investigated the prejunctional and postjunctional inhibition of adrenergic transmission in the rat isolated anococcygeus muscle by cimetidine.

Najbar et al. (1996) found that smooth muscle cells in the rat anococcygeus muscle are endowed with two distinct P-2-purinoreceptors which subserve contractions.

Pettibone et al. (1993) examined the inhibitory potency and selectivity of an oxytocin antagonist against oxytocin-stimulated contractions if the **mouse** anococcygeus muscle.

Dehpour et al. (1993) and Radjaee et al. (1996) used isolated anococcygeus muscles from **rabbits** and found an extremely regular activity induced by methoxamine or clonidine.

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A.1.2.6 β_1-Sympatholytic activity in isolated guinea pig atria

PURPOSE AND RATIONALE

The β-agonist isoprenaline (isoproterenol) induces an increase in the frequency and force of contraction of spontaneously beating isolated right atrias and potentiates contractions of electrically stimulated isolated left atria. Drugs with β-sympatholytic activity inhibit these isoprenaline-induced effects. β-receptor blocking activity of drugs can be evaluated in isolated right (a) and left (b) guinea pig atria. Since the heart contains predominantly β_1-adrenoreceptors, β_1-blocking activity is assessed by this test.

PROCEDURE

Pirbright White guinea pigs of either sex weighing 250–300 g are used. The animal is sacrificed by stunning and exsanguination. The heart is removed, the right or the left atrium is cut off and mounted in a 50 ml organ bath with a preload of 100 mg. The Krebs-Henseleit solution is maintained at 32 °C and aerated with 95% O_2/5% CO_2. Contractions are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronik, Freiburg).

Right atrium

After an equilibration period of 30 min, isoprenaline is administered into the organ bath to potentiate inotropy and frequency of the isolated right atrium. Cumulative doses of isoprenaline are added starting from a concentration of 0.05 µg/ml; consecutive doses are administered at 3 min intervals.

When a stable maximum plateau of the effect is achieved, the organ bath is thoroughly flushed for 1 min; flushing is repeated twice 5 and 20 min later. The whole procedure is repeated with the same isoprenaline concentrations (control baseline values = 100%).

The test compound is then added into the organ bath and 5 min later, again isoprenaline is given at cumulative doses.

If the test compound has β-receptor blocking activity (β-sympatholytic),

1. higher isoprenaline concentrations are necessary to induce the same potentiation of inotropy and frequency or
2. at the same isoprenaline concentrations added as before, the increase in inotropy and frequency is reduced.

At the end of the experiment, again a cycle without test drug is performed.

Left atrium

The left atrium is stimulated by a square wave stimulator with 2 impulses/s at a voltage of 15 V and an impulse duration of 1 ms. After an equilibration period of 30 min, the β-agonist isoprenaline is added at concentrations of 0.05–0.1 mg/ml. The organ bath is then thoroughly flushed for 1 min. Flushing is repeated twice 5 and 20 min later. The whole procedure is repeated with the same cumulative isoprenaline concentrations (control baseline values = 100%) and flushing procedure.

When a stable plateau of contractions is achieved, the test compound is added into the organ bath and 3 min later, isoprenaline is added again at cumulative concentrations.

If the test compound has β-receptor blocking activity (β-sympatholytic), the isoprenaline-induced effects are inhibited.

In addition, refractory period is determined before and after drug administration.

EVALUATION

a) Percent inhibition of (a) isoprenaline-induced or (b) electrically-induced and isoprenaline-potentiated increased inotropy and frequency by test drug is calculated as compared to pre-drug activity (= 100%).
b) Percent change in refractory period is calculated.

IC_{50} values are determined from the individual dose-response curves.

Statistical evaluation is performed by means of the paired *t*-test.

Standard compounds:

- propranolol HCl,
- amrinon,
- nifedipine,
- and milrinone.

MODIFICATIONS OF THE METHOD

A detailed description of the use of isolated atrial preparations has been given by Levy (1971).

Instead of the right atrium, Doggrell and Hughes (1986) used the isolated right ventricle of the rat for the assessment of the β-adrenoreceptor blocking activity of propranolol and investigated the competitive nature of the isoproterenol antagonism at various doses with Schild-plot analysis. Doggrell (1988) used the isolated left atria of the rat for simultaneous assessment of membrane-stabilizing and β-adrenoreceptor blocking activity.

Berthold et al. (1990) described a method for testing cardiotonic sodium channel activators in isolated, electrically stimulated left guinea pig atria after potassium depolarization.

Olson et al. (1995) studied the function of isolated rat left atria and papillary muscles and quantified the voltage-response relationship between punctate and field electrical stimulation after pretreatment with reserpine or β-blockers.

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A.1.2.7 β_2-Sympatholytic activity in the isolated tracheal chain

PURPOSE AND RATIONALE

Contraction of bronchial smooth muscle is induced by the cholinergic agonist carbachol. The carbachol effect can be antagonized by the β-agonist isoprenaline (isoproterenol). A compound has β-sympatholytic activity if the spasmolytic action of isoprenaline is inhibited. The ß-sympatholytic effect of drugs can be evaluated in an *in vitro*-model. Since the trachea contains predominantly β_2-adrenoreceptors, β_2-blocking activity can be assessed by this test.

PROCEDURE

Male Pirbright White guinea pigs weighing 250–300 g are used. The animals are sacrificed by stunning and exsanguination. The trachea is removed and cut into individual rings. Six rings are connected in series by means of short loops of silk thread. The tracheal chain is mounted in an 50 ml organ bath with a preload of 1 g for isotonic registration. To the nutritive solution (Tyrode) containing ascorbic acid and 1.0 g/L glucose, the α-receptor blocking agent phentolamine (0.1 µg/ml) and the spasmogen carbachol (80 ng/ml) are added. The solution is maintained at 34 °C and aerated with 95% O_2, 5% CO_2

Experimental course

After an equilibration period of 30 min, cumulative doses of 10^{-10} to 10^{-7} M of the spasmolytic agent isoprenaline are added. When maximal relaxation is obtained, the organ bath is flushed and the procedure repeated. After the two control relaxations with isoprenaline, the tissue is rinsed thoroughly and the first dose of the test compound is administered. Three min later, cumulative doses of isoprenaline are administered as before. Following a 10 min washout and recovery period, the next dose of the test compound is given. Up to 10 drug concentrations can be tested with one organ.

Standard compounds:
- propranolol
- practolol

EVALUATION

Percent inhibition of isoprenaline-induced relaxation under drug treatment is calculated compared to maximal relaxation induced by isoprenaline alone (control = 100%).

A competitive antagonism of test compound is evaluated and can be quantitated from the dose-response curve.

MODIFICATIONS OF THE METHOD

O'Donnell and Wanstall (1980) used guinea pig tracheal preparations, where K^+-depolarization was achieved by replacing all the Na^+ in Krebs solution by an equivalent amount of K^+ causing a sustained contraction of the preparations. A dose-dependent relaxation effect of isoprenaline could be obtained provided that the preparations were repolarized by washing in normal Krebs solution between curves. pA_2 values were in good agreement with values obtained in other types of tracheal preparations.

Guinea pig superfused trachea and dispersed tracheal cells have been used by Buckner et al. (1995) to compare the effects of isoproterenol and forskolin on immunologic and nonimmunologic histamine release.

The rat portal vein has been recommended as model for assessment of β_2 adrenoceptor blocking activity of drugs by Doggrell (1990).

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A.1.2.8 Angiotensin converting enzyme inhibition in the isolated guinea pig ileum

PURPOSE AND RATIONALE

The angiotensin-converting enzyme (ACE) is responsible for the formation of the active angiotensin II from the inactive angiotensin I. The same enzyme is responsible for the degradation of the active peptide bradykinin to inactive products. ACE activity can therefore be measured in two ways: activity of the newly formed angiotensin II and inhibition of the activity of bradykinin. ACE inhibition results in decreased activity of the precursor angiotensin I and potentiation of the bradykinin effect. The guinea pig ileum contracts in response to both peptides, angiotensin II and bradykinin, and can be used for quantitative determination of ACE inhibiting activity.

PROCEDURE

Guinea pigs of either sex weighing 300 to 500 g are used. They are sacrificed by stunning and exsanguination. The abdomen is opened with scissors. Just distal to the pylorus, a cord is tied around the intestine which is then severed above the cord. The intestine is gradually removed, and the mesentery is being cut away as necessary. When the colon is reached, the intestine is cut free. Below the cord, the intestine is cut halfway through, so that a glass tube can be inserted. Tyrode's solution is passed through the tube and the intestine until the effluent is clear. Mesentery is cut away from the intestine that was joined to the colon. Pieces of 3 cm length are cut. Preferably, the most distal piece is used being the most sensitive one. This piece is fixed with a tissue clamp and brought into an organ bath with Tyrode's solution at 37 °C being oxygenated with O_2. The other end is fixed to an isometric force transducer (UC 2 Gould-Statham, Oxnard USA). Responses are recorded on a polygraph.

Angiotensin I antagonism

After an equilibrium time of 30 min, angiotensin I is added in a concentration of 10 ng/ml bath solution. The force of contraction is recorded and the angiotensin I dosage is repeated once or twice until the responses are identical. Then the potential ACE inhibitor is added. After 5 min incubation time, again angiotensin I is added. The contraction is diminished depending on the activity of the ACE inhibitor.

Bradykinin potentiation

Pieces of guinea pig ileum are prepared as described before. After an equilibrium time of 30 min bradykinin is added in a concentration of 15 ng/ml bath solution. The force of contraction is recorded and bradykinin additions are repeated once or twice until the response is identical. Then the potential ACE inhibitor is added. After 5 min incubation time, again bradykinin is added. The contraction is potentiated depending on the activity of the ACE inhibitor.

EVALUATION

Angiotensin I antagonism

The contraction after addition of the ACE inhibitor is expressed as percentage of contraction without the ACE inhibitor. Using various doses of the ACE inhibitor IC_{50} values (concentrations inducing 50% inhibition) are calculated. As standards ramipril, enalapril, and captopril are used.

Bradykinin potentiation

The increase of the contraction after addition of the ACE inhibitor is expressed as percentage of contraction without the ACE inhibitor. As standard ramipril is used.

CRITICAL ASSESSMENT OF THE METHOD

The classical method of the isolated guinea pig ileum has been proven to be a reliable method for screening of potential ACE inhibitors.

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A.1.2.9 Contractile and relaxing activity on isolated blood vessels including effects of potassium channel openers

PURPOSE AND RATIONALE

The contractile process within the vascular smooth muscle results from an increase in the concentration of intracellular Ca^{2+}. Inhibition of vasoconstriction occurs by addition of calcium antagonists or by removal of extracellular calcium. The vasorelaxing effects of compounds can be tested in isolated rodent arteries (pulmonary artery, thoracic aorta). Arterial rings or strips with or without endothelial lining are contracted with different agents, e.g., extracellular K^+ and Ca^{2+}, the α-adrenoceptor agonists phenylephrine and noradrenaline, the Ca^{2+} ionophore A23187 or the thromboxane receptor agonist U46619. Compounds with vasodilating activity antagonize the induced contractions.

Potassium channel openers such as cromakalin, nicorandil, pinacidil or HOE 234 induce relaxation of contracted smooth musculature (Bolton et al. 1998). These effects are explained by data from patch clamp technique and ion flux experiments as well as by antagonism against potassium channel blockers. They indicate the potential use as antihypertensive and anti-asthmatic drugs (Hamilton and Weston 1989; Edwards and Weston 1990, 1993; Weston and Edwards 1992). The studies are complicated by the high diversity of potassium channels including ATP-sensitive, voltage-sensitive and Ca^{2+}-activated channels (Mourre et al. 1986; Blatz and Magleby 1987; Ashcroft and Ashcroft 1990; Jan and Jab 1990; Pongs 1992; Wann 1993). Since each functional channel appears to consist of four different subunits, the possibility exists that there may be hundreds of different voltage-sensitive K channels, depending on their subunit composition. Ashcroft and Gribble (2000) discussed new windows on the mechanism of action of K_{ATP} channel openers.

Glibenclamide is an antagonist of the ATP-modulated K^+ channel allowing the localization of the binding sites (Eltze 1989; French et al. 1990; Mourre et al. 1990; Miller et al. 1991).

PROCEDURE

Male Pirbright White guinea pigs weighing about 400 g, or Chinchilla rabbits weighing about 3.5 kg, or Sprague-Dawley rats weighing 250–400 g are used as donor animals. The tested vessels are the thoracic aorta or the arteria pulmonalis.

Materials and solutions

Physiological salt solutions (PSS) [mM]

	PSS I	PSS II	PSS III
NaCl	122	112	72 (92)
KCl	5.0	5	40 (20)
$CaCl_2$	1.2	–	–
$MgSO_4$	0.56	0.56	0.56
KH_2PO_4	1.2	1.2	1.2
$NaHCO_3$	25	25	25
EDTA	–	0.2	–
glucose	12	12	12

Contracting agents

$K^+ + Ca^{2+}$	40 mM + 0.5 mM
$K^+ + Ca^{2+}$	20 mM + 0.5 mM
U 46619 (thromboxane A_2 analogue)	1 μM
A 23187 (calcium ionophore)	5 μM
noradrenaline	1 μM
phenylephrine	0.1 μM
acetylcholine	1 μM
oxyhemoglobin	10 μM
methylene blue	10 μM

Animals are sacrificed by stunning and exsanguination. At least 4 isolated organs are tested per drug. The heart and the pulmonary artery are quickly removed and immersed in PSS I at room temperature. The artery is dissected into rings and endothelial cells are removed by gently rubbing the intimal surface. Spirally cut strips of 15–20 mm length and 1–1.5 mm width are suspended at a resting force of 380 mg in an organ bath containing 20 ml oxygenated (95% O_2, 5% CO_2) PSS I at 37 °C. Changes in length are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronic, Freiburg).

To test the effect of compounds on vessels with intact endothelial lining, the thoracic aorta of rats is isolated and dissected free from surrounding tissue. Rings of 3 mm width are cut and suspended in the organ bath containing PSS I. Isometric force is measured with a force transducer (UC-2, Gould-Statham, Oxnard, USA) under a resting tension of 500 mg.

The functional integrity of the endothelium is tested before drug administration. One μM acetylcholine in the organ bath should result in a transient relaxation.

After an equilibration period of 1 h, contraction of each vessel strip or ring is induced by addition of one of the contracting agents into the organ bath.

To induce contractions of potassium-depolarized vessels, three different PSS solutions are used (PSS I for 30 min, PSS II for 3×15 min and PSS III). Contraction is induced in the presence of PSS III by adding 0.5 mM Ca^{2+} into the organ bath.

When a stable plateau of contraction is achieved, cumulative concentrations of the test compound are added into the organ bath to obtain drug-response curves. Consecutive concentrations are added either at 1 h-intervals or when the response of the previous dose has reached a steady state level.

In order to study the time course of relaxation and the duration of action, only one concentration is tested.

To test whether the mechanism of action of a vasorelaxing agent is related to the liberation of nitric oxide, methylene blue or oxyhemoglobin (10 μM) are added to the organ bath 15–30 min prior to the cumulative administration of the test compound. Methylene blue or oxyhemoglobin block selectively NO induced relaxation.

EVALUATION

Mean values of relaxation ±SEM are calculated. The height of contraction before the first drug administration is taken as 100%.

IC^{50} values are determined from the individual dose-response curves. IC^{50} is defined as the dose of drug leading to a 50% relaxation of the contraction induced by KCl or other agonists.

Statistical evaluation is performed by means of the *t*-test.

MODIFICATIONS OF THE METHOD

Calderone et al. (1996) compared four **rat** aortic preparation (single ring, spiral strip, zig-zag strip, and multiple ring) on the basis of responses to noradrenaline and acetylcholine. They recommended the multiple ring preparation as the most suitable of all four for the study of vasoactive drugs because of the reproducibility of both contracturant and relaxing responses.

Kent et al. (1982) used rat aortic strips contracted to a stable tension by either phenylephrine or barium chloride for comparison of vasodilators.

Wilson et al. (1988) studied in isolated rings of rat aorta precontracted with noradrenaline the antagonism of glibenclamide against the vasorelaxation induced by cromakalin.

Nishimura and Suzuki (1995) tested the contractile responses to 5-HT in basilar arteries, superior mesenteric arteries and thoracic aortas from **stroke-prone spontaneously hypertensive rats** in comparison to normal Wistar-Kyoto rats and found that that the hyperresponsiveness to 5-HT is mediated by different 5-HT receptor subtypes.

Fouda et al. (1991) used **the isolated tail arteries from rats**. Differences of the vasoconstrictor response to potassium and norepinephrine between tail arteries from spontaneously hypertensive, renovascular hypertensive, and various strains of normotensive rats were found.

Hamilton et al. (1986), Dacquet et al. (1987) studied the effects of calcium entry blockers in **rat portal vein**.

Bråtveit and Helle (1984) studied the inhibition of vascular smooth muscle by vasoactive intestinal peptide (VIP) in the isolated rat portal vein.

Shetty and Weiss (1987) studied the inhibition of spontaneous rhythmic movements and norepinephrine-induced tension responses in the rat portal vein.

Smith et al. (1993) tested the ability of C-terminally truncated fragments of human α-calcitonin gene-related peptide to relax **mesenteric arteries** precontracted with norepinephrine

Chen et al. (1996) studied the contractile effects of noradrenaline and neuropeptide Y given alone or in combination on isolated rat mesenteric resistance vessels.

Gurden et al. (1993) used **guinea pig** aorta relaxation for functional characterization of adenosine receptor types.

Eltze (1989) studied the antagonism of glibenclamide against potassium channel openers in the isolated **guinea pig pulmonary artery**.

Szentmiklósi et al. (1995) used circular segments from the proximal part of the main pulmonary artery of guinea pigs to study contractile and relaxant effects of adenosine receptors.

Pikkers and Hughes (1995) examined the effect of hydrochlorothiazide on intracellular calcium concentration $[Ca^{2+}]_i$ and tone in **guinea pig mesenteric arteries**. Vessels were mounted on a microvascular myograph and loaded with the Ca^{2+}-sensitive fluorescent dye, Fura-2.

Meisheri et al. (1990) recommended the use of the isolated **rabbit mesentery artery** as a sensitive *in vitro* functional assay to detect K^+-channel-dependent vasodilators.

Mironneau and Gargouil (1979) studied the influence on electrophysiological and mechanical parameters of longitudinal smooth muscle strips isolated from **rabbit portal vein** by means of a double sucrose gap method associated with a photoelectric device for recording contractions.

McBean et al. (1986, 1988) used isolated segments of the arteria basilaris of **pigs** to detect compounds with antivasoconstrictive properties. Contraction is elicited by $PGF_{2\alpha}$, serotonin or norepinephrine. Specimens are

obtained from adult pigs (strain: Deutsche Landrasse) within 30 min after slaughter from the local slaughter house and stored in nutritive solution. The vessels are trimmed to a length of 4 mm, and the segments are suspended between 2 L-shaped metal hooks in a bath containing 20 ml modified Krebs Henseleit solution (NaCl 148 mM, KCl 5.4 mM, $CaCl_2$ 2.2 mM, $NaHCO_3$ 12 mM, glucose 12 mM). The bath solution is maintained at 37 °C and continuously gassed with carbogen to produce a resulting pH of 7.35–7.45. The preparation is incubated under a tension of 37.28 mN (optimal passive load producing the largest contractile response to 3×10^{-6} M $PGF_{2\alpha}$). Following a 60 minute stabilization period, the vessels are sensitized with 30 mM KCl for 10 min. The vessels are washed for 1 minute, and allowed to recover for 30 min with additional 1 minute washes at 15 and 30 min. Thereafter, contractions of the vessels are induced by adding $PGF_{2\alpha}$ at 3×10^{-6} M. The contractile response is allowed to plateau, then the test compound is administered at cumulative doses.

For each test compound a dose-response curve is recorded. The EC_{50} is obtained graphically or by means of a Hill plot. The EC_{50} is defined as the dose of drug producing half maximal response.

Werner et al. (1991) studied the vascular selectivity of calcium antagonists using **porcine** isolated ventricular trabeculae and right **coronary arteries**.

Merkel et al. (1992) used isolated porcine coronary artery rings precontracted with prostaglandin $F_{2\alpha}$ to demonstrate the vasorelaxant activity of an A_1-selective adenosine agonist.

Miwa et al. (1993) compared the effect of a K^+-channel opener with cromakalim, nitroglycerin and nifedipine on endothelin-1-induced contraction of porcine coronary artery.

Satoh et al. (1993) investigated in isolated porcine large coronary arteries whether or not the vasorelaxant actions of nicorandil and cromakalim would be selective using seven different vasoconstrictor agonists.

Yokoyama et al. (1994) studied the vasodilating mechanisms of several pyridinecarboximidamide derivatives in isolated porcine coronary arteries.

Makujina et al. (1995) described a procedure that facilitates the eversion of vascular smooth muscle. Vascular segments of porcine coronary artery, approximately 2 cm in length, were sutured to portions of polyethylene tubing inserted into the lumen of the vessel. After being secured and stabilized by the tubing, the vessel was everted while immersed in physiological buffer. Intracellular calcium concentrations (measured by fura-2AM fluorometry) and tension were registered simultaneously in everted rings denuded of endothelium.

Izumi et al. (1996) tested a K^+-channel opener and related compounds in isolated porcine coronary arteries contracted with 25 mM KCl.

Frøbert et al. (1996) described impedance planimetry as a new catheter-based technique to measure porcine coronary artery pharmacodynamics and compared the results with the commonly used wire-mounted isometric tension technique after *in vitro* application of nifedipine in various concentrations. A four-electrode impedance measuring system was located inside a 12-mm long balloon which was introduced into 3–4 cm long segments of the left anterior descending coronary artery obtained from 70–90-kg Danish Landrace-Yorkshire pigs.

Hamel et al. (1993) dissected segments (3–4 mm long) of temporal ramifications of the middle cerebral artery from **bovine** brains and mounted them between two L-shaped metal prongs in a tissue bath containing Krebs-Ringer solution at 37 °C. Changes in muscle tension were measured by a force displacement transducer and recorded on a polygraph. Several 5-HT receptor agonists were tested for their ability to induce vasoconstriction and their potencies were compared to that of 5-HT. The authors concluded that bovine pial arteries appear to be the best available model for the human cerebrovascular $5\text{-}HT_{1D}$ receptor.

De la Lande et al. (1996) used isolated segments from proximal (4.5 mm i.d.) and distal (0.5 mm i.d.) bovine coronary arteries and found a heterogeneity of response to glyceryl trinitrate.

The isolated **human** coronary artery was used to study the vasoconstriction by acutely acting antimigraine drugs (Saxena et al. 1996a,b, 1997)

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A.1.2.10 Isolated guinea pig ureter

PURPOSE AND RATIONALE

The isolated guinea pig ureter shows phasic-rhythmic contractions after addition of KCl to the organ bath. Inhibition of this effect can be explained as a modulation of potassium channels.

PROCEDURE

Male unfasted guinea pigs weighing 400–500 g are sacrificed and both ureters removed immediately without the part directly connected to the pelvis in order to exclude the pacemaker region responsible for spontaneous activity. Each segment of 2 cm length is placed in a Petri dish containing Tyrode solution at 37 °C, freed of surrounding connective tissue and then suspended at a baseline tension of 0.5 p in a 25 ml organ bath containing Tyrode solution at 37 °C being aerated with 5% CO_2/95% O_2, pH 7.4. Contractions are measured isometrically using Gould/Statham UC 2 transducers. After a 15 min equilibration period, KCl is added to the bath in a final concentration of 3×10^{-2} Mol/l and left in the bath for 2 min. KCl induces a constant series of phasic-rhythmic contractions without a rise in baseline tone. Subsequent washing causes the immediate disappearance of the rhythmic contractions. This addition of KCl is repeated and the values of these two experiments are used as initial values. The antagonistic activity is studied by addition of the test drug one min prior to the KCl challenge. Percentage of the following parameters are determined: mean height of contractions, frequency of contractions and the product of mean height and frequency of contractions. For interaction studies the potassium channel blocker glibenclamide 10^{-6} mol/l is added 1 min prior to the test drug.

EVALUATION

Arithmetic means and standard deviations of the data are calculated and compared with initial values using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The isolated guinea pig ureter stimulated with KCl can be used for studies on the modulation of potassium channels.

MODIFICATIONS OF THE METHOD

Yoshida and Kuga (1980) recorded electrical activities in a preparation consisting of the pelvic region and the upper ureter of the guinea pig. Train field stimulation of the pelvic region evoked a train of nerve action potentials followed by a multiphasic smooth muscle action potential after a latency of about 2.5–8.0 s. This smooth muscle response was abolished by tetrodotoxin and dibucaine, and also by cholinergic blocking agents.

The effects of veratridine and of yohimbine on the efflux of norepinephrine from electrically stimulated guinea pig ureters were studied by Kalsner (1992).

Maggi and Giuliani (1994) studied the excitability and refractory period of the guinea pig ureter to electrical field stimulation.

Roza and Laird (1995), Laird and Cervero (1996) studied the pressor responses to distension of the ureter in anesthetized rats as a model of acute visceral pain.

A simple method for measurement of ureteric peristaltic function *in vivo* in anesthetized rats was published by Kontani et al. (1993).

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A.1.2.11 Isolated corpus cavernosum

PURPOSE AND RATIONALE

The isolated corpus cavernosum of rabbits has gained interest as pharmacological model since selective inhibitors of cyclic guanosine monophosphate (cGMP) phosphodiesterase type 5 (PDE5) were found to be effective in the treatment of erectile dysfunction in man (Ballard et al. 1996; Jeremy et al. 1997; Chuang et al. 1998; Liu et al. 1998; Turko et al. 1999; Wallis 1999; Wallis et al. 1999; Stief 2000).

PROCEDURE

Male New Zealand White rabbits weighing 3–4 kg are sedated with an intramuscular injection of 25 mg/kg ketamine + 6 mg/kg xylazine. Anesthesia is maintained by intravenous injection of 25 mg/kg nembutal. The penis is removed at the level of the attachment of the corporal bodies to the ischium. The corpus cavernosum (total length about 20 mm) is sharply dissected from tunica albuginea and two longitudinal strips with unstretched length about 10 mm are made from the proximal, more muscular portion.

Corporal strips are placed in organ baths containing 10 ml Tyrode's buffer (NaCl 124.9 mmol/l, KCl 12.5 mmol/l, $MgCl_2 \cdot 6\,H_2O$ 0.5 mmol/l, $NaH_2PO_4\,H_2O$ 0.4 mmol/l, $CaCl_2$ 1.8 mmol/l and glucose 5.5.mmol/l) at 37 °C. Each tissue is equilibrated with a mixture of 95% O_2 and 5% CO_2 at pH 7.4. One end of each strip is connected to a force displacement transducer, and changes in muscle tension are measured and recorded with a polygraph. After zeroing and balancing transducers and strip chart, 2.0 g of tension is placed on each strip, and the strips are allowed to equilibrate for 30 min.

Each strip is prestimulated with 10 μM phenylephrine, then relaxed by electrical field stimulation with square wave pulses of 80 V, 1 ms duration at 2–16 Hz frequency. Then sodium nitroprusside (0.01–100 μM) is added ad NO donor. Finally, the standard (sildenafil 1 nM to 1 μM) or the test compound is added.

EVALUATION

The dose-dependent increase of relaxation after test compound and standard is measured. From dose-response curves activity ratios can be calculated.

MODIFICATIONS OF THE METHOD

Wallis et al. (1999) studied the inhibition of human phosphodiesterases PDE1 to PDE6 by sildenafil in various tissues, such as cardiac ventricle, corpus cavernosum, skeletal muscle and retina.

Park et al. (1997) reported functional characterization of angiotensin II receptors in rabbit corpus cavernosum.

Yildirim et al. (1997) investigated the effects of castration and testosterone on the constricting effect of phenylephrine and endothelium-dependent and -independent relaxing effects of different agonists in the corpus cavernosum of male rabbits.

Liu et al. (1998) analyzed the pharmacological effects of *in vitro* ischemia on rabbit corpus cavernosum.

Gupta et al. (1998) found that activation of G_i-coupled postsynaptic α_2-adrenoceptors causes contraction of smooth muscles in the corpus cavernosum of rabbits.

Teixeira et al. (1998) used a bioassay cascade to study the effect of *Tityus serrulatus scorpion* venom on the rabbit isolated corpus cavernosum.

Cellec and Moncada (1998) used the clitoral corpus cavernosum of female rabbits to study the role of nitrergic neurotransmission in non-adrenergic non-cholinergic relaxation responses.

The isolated corpus cavernosum of **rats** has been used by Tong and Cheng (1997), of **mice** by Gocmen et al. (1997), of **dogs** by Hayashida et al. (1996), Comiter et al. (1997), of **monkeys** by Okamura et al. (1998), of **horses** by Recio et al. (1997).

Studies in isolated **human** corpus cavernosum were performed by Cellek and Moncada (1997), Omote (1999), Lin et al. (2000), Stief et al. (2000).

In vivo studies measuring intracavernous pressure in **rats** were performed by Ari et al. (1996), Chan et al. (1996), Moody et al. (1997), Reilly et al. (1997), Chang et al. (1998), Mills et al. (1998).

In vivo studies on penile erection were performed in **cats** by Champion et al. (1997).

Intracavernous pressure was measured *in vivo* in anesthetized **dogs** by Ayajiki et al. (1997), Sarikaya et al. (1997), Carter et al. (1998).

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A.1.3 Cardiovascular analysis *in vivo*

A.1.3.1 Hemodynamic screening in anesthetized rats

PURPOSE AND RATIONALE

The test is used to detect the effect of compounds on blood pressure and heart rate of anesthetized rats and to check for possible interference with adrenergic receptors. Antihypertensive agents with different mechanisms of action can be detected with this test.

PROCEDURE

Male Sprague-Dawley rats weighing 250–400 g are used. At least 2 animals are necessary for screening of one compound. The rats are anesthetized by intraperitoneal injection of 8 ml/kg of a solution of 8% urethane and 0.6% chloralose. The trachea is cannulated to facilitate spontaneous respiration. Body temperature is maintained at 38 °C by placing the animal on a heating pad.

The left femoral vein is cannulated for drug administration, which is standardized to injections of 0.2 ml/100 g body weight over a period of 1 min. For measurement of hemodynamic parameters and for intra-arterial administration of test compound, a cannula is inserted retrogradely into the right carotid artery. The tip of the catheter is positioned close to the origin of the subclavian artery. This allows most of the injected substances to reach the CNS via the vertebral artery before going into the general circulation.

For continuous monitoring of blood pressure (systolic and diastolic pressure) and heart rate, the catheter is connected to a pressure transducer (Statham DB 23).

When stable hemodynamic conditions are achieved for at least 20 min (control values), test boli of adrenaline (1 mg/kg) and isoprenaline (0.25 mg/kg) are administered. When baseline values are again established, increasing doses of the test substance (0.01, 0.1, 3.0 mg/kg) are given intra-arterially. In case of no effect, the interval between successive doses is 15 min, otherwise 60 min. To check for α- or β-blocking activity, adrenaline and isoprenaline administration is repeated after injection of the highest dose of test compound. If the test compound shows no effect, a standard antihypertensive compound is administered for control purpose.

Hemodynamic parameters are recorded continuously during the whole experiment.

EVALUATION

Changes in blood pressure and heart rate after drug administration are compared to control values obtained during the 20 min pre-drug period.

Maximal changes in BP and HR and duration of the effect are reported.

The results are scored relative to the efficacy of standard compounds for the degree of the effect and the duration of the effect.

Statistical significance is not tested because of the small number of animals used ($n = 2$, sometimes 3 or 4) but larger numbers of animals have to be used for quantitative evaluation.

CRITICAL ASSESSMENT OF THE METHOD

Due to the administration of the test compounds via the right common carotid artery not only peripherally acting vasodilators and neuron blockers but also compounds affecting the blood pressure regulating mechanisms in the CNS are detected. Bolus injections of adrenaline and isoprenaline reveal possible α- or β-antagonistic effects.

Standard data:
The following compounds at the doses indicated lead to a strong decrease in blood pressure:

• Clonidine	0.008	mg/kg
• Dihydralazine	1.0	mg/kg
• Phentolamine	3.0	mg/kg
• Prazosin	0.1	mg/kg
• Propranolol	1.0	mg/kg
• Urapidil	1.0	mg/kg
• Verapamil	0.1	mg/kg

MODIFICATIONS OF THE METHOD

A procedure for differential intra-arterial pressure recordings from different arteries in the rat was described by Pang and Chan (1985).

DeWildt and Sangster (1983) described the evaluation of derived aortic flow parameters measured by means of electromagnetic flowmetry as indices of myocardial contractility in anesthetized rats.

Using a special Millar ultraminiature catheter pressure transducer and a thermodilution microprobe, Zimmer et al. (1987, 1988) measured right ventricular functional parameters in anesthetized, closed-chest rats.

Veelken et al. (1990) published improved methods for baroreceptor investigations in chronically instrumented rats.

Salgado and Krieger (1988), de Abreu and Salgado (1990), Da Silva et al. (1994) studied the function of the **baroreceptor reflex** in thiopental anesthetized rats. The left aortic nerve was isolated and supported by a bipolar stainless steel electrode and carefully insulated with silicone rubber. Carotid pressure was recorded simultaneously with aortic nerve discharges on an oscilloscope and monitored with a loudspeaker.

King et al. (1987) developed a cross circulation technique in rats to distinguish central from peripheral cardiovascular actions of drugs. The right common carotid arteries were ligated, and the left common carotid arteries and left and right external jugular veins of two phenobarbital-anesthetized rats were connected with polyethylene tubing so that peripheral blood from one rat, A, supplied the head of another rat, B, and then returned to the body of A, and vice versa, for peripheral blood from rat B. Each rat was artificially ventilated with O_2, the chest was opened, and both subclavian arteries were ligated. Prior to the ligation of the subclavian arteries, blood flow from rat A supplied its own brain and both brain hemispheres but not the brain stem of rat B. Following subclavian artery ligation, blood flow from rat A did not supply A's brain, but supplied both hemispheres and brain stem of rat B. The head of each rat was, therefore, rendered dependent on the carotid arterial blood supply from another rat. This rat cross-circulation preparation can be used to separate the central and peripheral cardiovascular actions of drugs.

Zavisca et al. (1994) studied the hypertensive responses to defined electrical and mechanical stimuli in anesthetized rats. Rats were given etomidate, 3.8 mg/kg/h intravenously following carotid artery and jugular vein cannulation. At 15 min after beginning the infusion, 4 types of noxious stimuli were administered sequentially at 1-min intervals: Type 1: Square electrical waves 125 cps, 1.6 ms, 2-s duration, varying current from 0.4 to 12 mA; Type 2: A single 10-mA electrical stimulus, 5-s train duration; Type 3: Tail clamping; Type 4: Skin incision. After each stimulus, maximum change in systolic blood pressure was measured. Graded electrical stimulation allowed the best quantitative evaluation of the hypertensive response to noxious stimuli.

Hyman et al. (1998) described a novel catheterization technique for the *in vivo* measurements of **pulmonary vascular responses** in rats. Male Charles River rats weighing 26–340 g were anesthetized and strapped in supine position to a fluoroscopic table. They breathed air enriched with oxygen through an endotracheal tube inserted by tracheostomy. Catheters were inserted into the femoral blood vessels. The venous catheters were passed to the right atrium under fluoroscopy. A F-1 thermistor catheter was passed from the left carotid artery into the ascending aorta under fluoroscopy, and a PE-50, 150-mm plastic catheter with a specially constructed curved tip was passed fluoroscopically from the left jugular vein into the main pulmonary artery. A plastic radiopaque 22-gauge catheter 100 mm in length with a curved tip was passed with a 0.025 mm soft-tip coronary guiding catheter from the right jugular vein through the right atrium to the inferior vena cava. The coronary soft-tip guide was then withdrawn. A specially curved 102.5-mm transseptal needle, 0.4 mm in diameter, was then passed though the catheter. Both the needle and catheter were withdrawn into the superior portion of the right atrium under fluoroscopic guidance so that the needle and catheter both rotated freely. With the rat in a slight left anterior oblique position, the catheter and needle were carefully rotated anteriorly to the intra-atrial septum. With gentle pressure, the catheter and needle can be felt and seen fluoroscopically to pass through the atrial septum. As the needle was withdrawn, the curve of the catheter permitted passage of the tip into the vein draining either the left or right lower lobe. The catheter was carefully positioned near the pulmonary venoatrial junction and fixed in place. Mean pressures in the femoral artery, pulmonary artery and pulmonary vein at the venoatrial junction were measured with pressure transducers and recorded on a polygraph. Cardiac output was obtained in triplicate by delivering 0.1 ml normal saline at room temperature into the femoral venous catheter at the right venoatrial junction and determining thermodilution cardiac output with the thermistor catheter in the ascending aorta.

Hayes (1982) described a technique for determining contractility, intraventricular pressure, and heart rate in the **anesthetized guinea pig** by inserting a needle, attached to a pressure transducer, through the chest wall into the left ventricle.

Williams et al. (1995) used **castrated male ferrets** anesthetized by intramuscular injection of a mixture of 55 mg/kg ketamine and 4 mg/kg xylazine to measure the effects of a nonpeptidyl endothelin antagonist on endothelin-induced pressor responses.

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A.1.3.2 Blood pressure in pithed rats

PURPOSE AND RATIONALE

The pithed rat has been proposed for assessing pressor substances by Shipley and Tilden (1947). The preparation is frequently used to evaluate drug action on the cardiovascular system since this preparation is devoid of neurogenic reflex control that may otherwise modulate the primary drug effect.

PROCEDURE

Male rats weighing 250–350 g are prepared for pithing under halothane anesthesia. The left carotid artery is cannulated for blood pressure monitoring and blood sampling. Furthermore, the trachea and the right jugular vein are cannulated. The rats are pithed inserting a steel rod, 2.2 mm in diameter and about 11 cm in length, through the orbit and foramen magnum down the whole length of the spinal canal. Via the tracheotomy tube, the animals are ventilated with a small animal ventilation pump. Inspirated air is oxygen-enriched by providing a flow of oxygen across a T-piece attached to the air intake of the ventilation pump (Harvard Apparatus model 680). The rats are ventilated at a frequency of 60 cycles/min with a tidal volume of 2 ml/100 body weight. Thirty min after pithing, a 0.3 ml blood sample is withdrawn from the carotid cannula and immediately analyzed for pO_2, pCO_2, pH, and derived bicarbonate concentration using an automatic blood gas analyzer. By alterations of the respiratory stroke volume of the pump, the values are adjusted to: pCO_2 30–43 mm Hg, pH 7.36–7.50, pO_2 87–105 mm Hg.

Continuous registration of blood pressure and cardiac frequency (Hellige He 19 device and Statham P 23 Db transducer) is performed via the left carotid artery.

In order to measure α_1 and α_2 antagonism, first dose-response curves are registered using doses of 0.1–30 µg/kg i.v. phenylephrine (a selective α_1 agonist), and 1–1 000 µg/kg i.v. BHT 920, (a selective α_2 agonist). The test drug is administered intravenously and the agonist dose-response curves are repeated again 15 min later.

EVALUATION

If the curve of blood pressure response to the agonists is shifted, dose-response curves are plotted on a logarithmic probit scale and potency ratios are calculated.

MODIFICATIONS OF THE METHOD

Gillespie and Muir (1967) described a method of stimulating the complete sympathetic outflow from the spinal cord to blood vessels in the pithed rat by coating those parts of the pithing rod which lay in the sacral and cervical region of the spinal cord with high-resistance varnish to restrict stimulation to the thoraco-lumbar region. The steel rod is insulated with an adhesive throughout its length except for a 5 cm section which provides sufficient a stimulation area of the lower thoraco-lumbar nerves. For stimulating nerves fibers supplying exclusively the heart, a pithing rod is used which is insulated throughout its length except for a 0.5 cm section 7 cm proximal to the tip. The spinal cord is stimulated electrically using the pithing rod as the cathode and a hypodermic needle which is inserted under the skin near the right hind-limb, as the anode. Varying the intensity and/or the duration of the stimulation, dose-response curves can be registered which are altered after treatment with drugs.

Curtis et al. (1986) described an improved pithed rat method by mounting the preparation vertically with the head pointing downward resulting in considerably higher blood pressure and heart rate.

MacLean and Hiley (1988) studied the effect of artificial respiratory volume on the cardiovascular responses to an α_1- and α_2-adrenoceptor agonist in the air-ventilated pithed rat using microsphere technique and analysis of arterial blood gases and pH.

Trolin (1975) used decerebrated rats to study the clonidine-induced circulatory changes.

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A.1.3.3 Antihypertensive vasodilator activity in ganglion-blocked, angiotensin II supported rats

PURPOSE AND RATIONALE

The method is used to demonstrate direct vasodilator activity of potential antihypertensive agents. The experimental model is an anesthetized, ganglion-blocked rat whose blood pressure is maintained by an intravenous infusion of angiotensin II. The test allows to differentiate between centrally acting antihypertensives and peripheral vasodilators.

PROCEDURE

Male Wistar rats weighing 275–450 g are anesthetized with a combination of urethane (800 mg/kg) and chloralose (60 mg/kg) administered intraperitoneally in a volume of 10 ml/kg. Following induction of anesthesia, chlorisondamine (2.5 mg/kg) is injected into the peritoneal cavity to abolish sympathetic and parasympathetic nerve activity. The right femoral artery is cannulated to monitor blood pressure (Statham pressure transducer P23Db) and heart rate. Both femoral veins are cannulated to administer drugs or infuse angiotensin II. The trachea is intubated and animals are allowed to breathe spontaneously. Following a stabilization interval of 10–15 min, angiotensin II is infused at a rate of 0.25 or 3.5 µg/min in a volume equivalent to 0.05 ml/min (Harvard infusion pump).

After an increase of blood pressure, a new elevated steady-state pressure is established within 15–20 min. Drugs are subsequently injected intravenously over an interval of 3 min in a volume of 2 ml/kg. Mean arterial pressure is recorded on a polygraph at 5, 10, 15, 20 and 30 min after initiation of drug administration. Seven to 9 animals are used for each drug and dose level studied.

α-adrenoreceptor blockade can be determined in ganglion-blocked rats. Pressor responses to graded doses of phenylephrine injected intravenously are obtained before and 15 min after administration of test compounds. Sufficient concentrations of phenylephrine have to be given to ensure a rise in mean arterial blood pressure of 50 mm Hg or more. Data obtained from 5 or 6 animals are averaged and resultant dose-response curves plotted. The dose of phenylephrine required to elicit a 50 mm Hg increase in mean arterial blood pressure is interpolated from dose response curves.

Standard data:
The following compounds are used as standards and, at the doses indicated, lower mean arterial blood pressure by about 50 mm Hg:

• Cinnarizine	3.0	mg/kg, i.v.
• Hydralazine	1.0	mg/kg, i.v.
• Minoxidil	10.0	mg/kg, i.v.
• Saralasine	0.03	mg/kg, i.v.
• Molsidomine	0.1	mg/kg, i.v.

EVALUATION

Mean values ±SEM are given for mean arterial blood pressure and heart rate. Changes of these parameters after drug administration are compared to control val-

ues obtained immediately before the application of the test compound. Statistical significance is assessed by means of the paired *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

A hypotensive response in this model appears to correlate more closely with antihypertensive activity in DOCA-salt hypertensive rats than does a vasodilator response in the perfused hind limb of anesthetized dogs and allows a distinction between central anti-hypertensive and vasodilators.

MODIFICATIONS OF THE METHOD

Santajuliana et al. (1996) developed a standard ganglionic blockade protocol to assess neurogenic pressor activity in conscious rats. Rats were instrumented with arterial and venous catheters for measurement of arterial pressure and heart rate and for administration of three different ganglionic blockers (trimethaphan, hexamethonium, and chlorisondamine).

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A.1.3.4 Blood pressure in conscious hypertensive rats (tail cuff method)

PURPOSE AND RATIONALE

Rats with spontaneous or experimentally induced hypertension are widely used for screening of potentially antihypertensive compounds. The indirect tail cuff method allows the determination of systolic blood pressure according to the following principle: The cuff is quickly inflated to well above suspected systolic blood pressure; the pulse will then be obliterated. Thereafter, pressure in the cuff is slowly released and, as the pressure falls below systolic blood pressure, the pulse will reappear. The method is analogous to sphygmomanometry in human and can be applied not only at the tail of awake rats but also in dogs and small primates. The indirect tail cuff method is widely used to evaluate the influence of antihypertensive drugs in spontaneously and experimentally hypertensive rats.

PROCEDURE

Male spontaneous hypertensive rats (Charles River) weighing 300–350 g or rats with experimentally induced hypertension are used.

Surgical procedure to induce renal hypertension

Male Sprague-Dawley rats weighing 80–100 g are anesthetized by intraperitoneal injection of 0.8 ml 4% chloralhydrate solution. Both kidneys are exposed retroperitoneally. To induce renal hypertension, a silver clip (0.2 mm diameter, 4 mm length) is placed onto both renal arteries, the kidneys are reposed and the wound is closed by suture.

Within 5–6 weeks, operated animals attain a renal hypertension with a systolic blood pressure (BPs) of 170–200 mm Hg (mean normal physiological BPs for rats is 100 mm Hg). Only animals with a BPs = 180 mm Hg are used for the tests.

Test procedure

The procedure is the same for spontaneously andexperimentally hypertensive rats. Groups of 6 animals are used per dose. The control group receives saline only. To reduce spontaneous variations in blood pressure, animals are adjusted to the experimental cage by bringing them into the restraining cage which is enclosed in a 31–32 °C measuring chamber 3–4 times before the start of the experiment for a period of 30–60 min.

To measure blood pressure, a tubular inflatable cuff is placed around the base of the tail and a piezo-electric pulse detector is positioned distal to the cuff. The cuff is inflated to approximately 300 mm Hg. As the pressure in the cuff is slowly released, the systolic pressure is detected and subsequently recorded on a polygraph.

The test substance is administered intraperitoneally or by gavage once per day over a period of 5 days. The usual screening dose of a new compound is 25 mg/kg. Blood pressure and heart rate measurements are taken at the following times:

day 1: predose and 2 h postdrug
day 3: predose and 2 h postdrug
day 5: predose, 2 h postdrug and 4 h postdrug.

Between measurements, animals are returned to their home cages.

Standard compounds:

- endralazine (3 mg/kg p.o.)
- nifedipine (3 mg/kg p.o.)
- urapidil (5 mg/kg p.o.)

EVALUATION

Mean values in systolic blood pressure before and after drug administration and the duration of the effect are determined. Percent decrease in systolic blood pressure under drug treatment is calculated. Statistical significance is assessed by the Student's *t*-test.

Scores for % decrease in systolic blood pressure and for the duration of the effect are allotted.

CRITICAL ASSESSMENT OF THE METHOD

The indirect tail-cuff method is being used in many laboratories with many modifications of the devices. Pfeffer et al. (1971) found a good correlation between values obtained with the indirect tail-cuff method and values measured directly with indwelling carotid arterial cannulae, whereas Buñag et al. (1971) reported a lack of correlation between direct and indirect measurement of arterial pressure in unanesthetized rats and Patten and Engen (1971) found difficulties to measure accurate systolic values at higher blood pressure. A good correlation between direct blood pressure data from the carotid artery in rats and readings with the tail cuff method was found by Matsuda et al. (1987) who developed a six-channel automatic blood pressure measuring apparatus with a highly sensitive photoelectric sensor for the detection of tail arterial blood flow and a microcomputer system for automatic measurement of systolic blood pressure and heart rate and for data acquisition and processing.

MODIFICATIONS OF THE METHOD

Details of the tail-cuff method in rats have been discussed by Stanton (1971).

Special equipment for measuring blood pressure in rats is commercially available (e.g., Rhema Labortechnik, Hofheim, Germany).

Widdop and Li (1997) described a simple versatile method for measuring tail cuff systolic blood pressure in conscious rats. A tail cuff consisting of a metal T-piece tube with latex rubber inside the tube is placed around the tail at the proximal end. A piezo-electric transducer (model MLT1010) is strapped to the ventral surface of the tail to record the pulse signal from the caudal artery and connected directly to a MacLab® data-acquisition system ADInstruments Pty Ltd.).

The tail-cuff method for measurement blood pressure has been adapted for dogs, monkeys (Wiester and Iltis 1976), and cats (Mahoney and Brody 1978).

Blood pressure can be measured from the hind leg of the rat using a leg cuff and a photoelectric cell situated at the dorsal surface of the foot (Kersten et al. 1947). When the leg is occluded, the foot swells and the amount of light striking the photocell is reduced. When the pressure in the cuff is released, the arterial blood flow is restored, the increase of foot volume is decreased and the amount of light transversing the paw increases.

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A.1.3.5 Direct measurement of blood pressure in conscious rats with indwelling catheter

PURPOSE AND RATIONALE

The method first described by Weeks (1960) allows the direct measurement of arterial pressure in conscious rats eliminating the influence of anesthesia on cardiovascular regulation.

PROCEDURE

Preparation of cannulae

In order to prepare the cannulae 7 cm and 12 cm long pieces are cut from PE 10 and PE 20 tubings respectively. A stylet wire is inserted into the PE 10 tubing and the PE 20 tubing is also slipped over the stylet wire. The ends of the tubings are heated in a current of hot air and fused together. Ridges are made to anchor the cannula in the animal's tissue. In order to make a ridge, the stylet wire is left inside the cannula and the cannula is heated in a fine jet of hot air. When the polyethylene at the point of heating becomes soft, the cannula is pressed slightly and thus a ridge is formed.

One ridge is formed at the PE 20 tubing, about 0.5 cm away from the junction with the PE 10 tubing, and 3 more ridges are formed on the PE 20 tubing at a distance of about 1 cm from each other, first one being situated about 3 cm away from the free end of the PE 20 tubing. The stylet wire is then removed from the cannula and the PE 10 portion of the cannula near the junction with the PE 20 tubing is wound around a glass rod with a diameter of 4 mm. Two rounds are made. Then it is dipped in a boiling water bath for about 5 s. When taken out of the bath, the cannula retains its circles, forming a spring-like structure.

Implantation of cannulae

Male Sprague-Dawley rats weighing about 300 g are used. The rat is anesthetized with 45 mg/kg pentobarbital i.p. The area of the neck and the abdomen are shaved and cleaned with 70% alcohol. The viscera are exposed through a midline abdominal incision. A segment of the abdominal aorta is exposed just above the bifurcation. A trocar is passed through the psoas muscles adjacent to this segment of the aorta, through the muscles of the back and under the skin until it emerges from the skin of the neck. Then the cannula is inserted into the trocar and the trocar is withdrawn from the body. The end of the cannula thus comes out from the neck, being anchored by silk sutures to the neck skin and to the psoas muscle. The cannula is filled with heparin solution and the end which is projecting out from the neck skin is blocked with a tight fitting stainless steel needle. Then the other end of the cannula is implanted into the aorta. The aorta is wiped with a cotton-tipped applicator stick above the bifurcation, occluded above this segment and punctured with a bent 27 gauge hypodermic needle. The tip of the PE 10 catheter is inserted through the needle and advanced up the aorta. The intestines are replaced and the wound sutured. The rats are allowed to recover for one week.

Measurement of blood pressure

The occluding stainless steel needle is removed and the cannula flushed with diluted heparin solution. The rat is placed in a small cage to restrict its movements, even so it is free to move. The cannula is connected to a Statham P 23 Db pressure transducer and blood pressure is recorded on a polygraph. Test drugs or standards are administered either subcutaneously or orally. Recordings are taken before and after administration of drug over a period of 1 h.

EVALUATION

Changes of blood pressure are measured for degree and duration. Five rats are used for each dose and compound. The maximal changes of each group are averaged and compared with the standard.

CRITICAL ASSESSMENT OF THE METHOD

Direct measurement of arterial blood pressure in unanesthetized rats originally introduced by Weeks (1960) has become a valuable and widely used tool in cardiovascular research.

MODIFICATIONS OF THE METHOD

A detailed description of a slightly modified Week's method has been given by Stanton (1971).

Improvements of the method for continuous direct recording of arterial blood pressure and heart rate in rats have been described by Buñag et al. (1971), Laffan et al. (1972), Buckingham (1976), Garthoff and Towart (1981), Garthoff (1983). A detailed description of permanent cannulation of the iliolumbar artery was given by Remie et al. (1990).

Wixson et al. (1987) described a technique for chronic catheterization of the carotid artery in the rat.

Prepared cannulas are commercially available (IRC Life Science, Woodland Hills CA).

A newer modification uses the access to the aorta via the common carotid artery (Linz et al. 1992). Rats are prepared under thiopental anesthesia with arterial PE-50 lines (Intramedic® from Clay Adams, USA). The lines are introduced into the ascending aorta via the right carotid artery for direct measurement of arterial blood pressure and into the jugular vein for i.v. application of test compounds. Both lines, filled with saline containing heparin, are surfaced on the neck. The animals are allowed to recover for at least 2 days. Blood pressure is monitored through Statham R P23 Db transducers connected to a recording device. During measurements the lines are kept open with counter current saline infusion at a rate of 1 ml/h.

Bao et al. (1991) placed one catheter via the right femoral artery in the abdominal aorta in rats for recording mean arterial pressure and two additional catheters via the left carotid artery into the descending aorta for application of bradykinin and bradykinin antagonists.

Arterial pressure was recorded in unanesthetized rats after induction of severe hypertension by complete ligation of the aorta between the origin of the renal arteries by Sweet and Columbo (1979).

Hilditch et al. (1978) described a device for the direct recording of blood pressure in conscious dogs.

Akrawi and Wiedlund (1987) described a method for chronic portal vein infusion in unrestrained rats. Hepatic drug metabolism can be studied by infusion into the portal vein and blood collection from the femoral vein.

Robineau (1988) described a method for recording electrocardiograms in conscious, unrestrained rats. Electrodes were implanted subcutaneously and a socket connector was sutured on the head of the animal. A flexible cord leading to a swivel collector was linked to an ECG amplifier.

Kurowski et al. (1991) reported on an improved method to implant, maintain, and protect arterial and venous catheters in conscious rats for extended periods of time.

Schenk et al. (1992) measured cardiac left ventricular pressure in conscious rats using a fluid-filled catheter.

Tsui et al. (1991) recommended a reliable technique for chronic carotid arterial catheterization in the rat.

Hagmüller et al. (1992) described a tail-artery cannulation method for the study of blood parameters in freely moving rats.

Liebmann et al. (1995) described an *in vivo* long-term perfusion system which is based on automated, computer-controlled high-frequency heparin (10 U/ml) flushing of a cannula inserted into the tail artery of freely moving rats.

Santajuliana et al. (1996) used conscious rats instrumented with arterial and venous catheters to assess neurogenic pressor activity after administration of ganglionic blockers.

Rezek and Havlicek (1975) described simple cannula systems for the infusion of experimental substances in chronic, unrestrained animals. A cannula with a removable cap is used for infusions into various parts of the digestive tract. Intravenous infusions can be performed through a closed system cannula which avoids a possible introduction of air into the circulation.

Kimura et al. (1988) described a method for chronic portal venous, aortic, and gastric cannulation to determine portal venous and aortic glucose and lactate levels in conscious rats.

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A.1.3.6 Cannulation techniques in rodents

PURPOSE AND RATIONALE

Cardiovascular pharmacology requires special techniques for catheterization and permanent cannulation of vessels. A few methods are described below.

A comprehensive literature survey on methods for vascular access and collection of body fluids from the laboratory rat was written by Cocchetto and Bjornsson (1983).

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A.1.3.6.1 Permanent cannulation of the jugular vein in rats

PURPOSE AND RATIONALE
Permanent cannulation of the jugular vein in rats in combination with a head attachment apparatus allowing easy connection of cannulae was first introduced by Steffens (1969). Modifications were described by Brown and Hege (1972), Nicolaidis et al. (1974) and by Dons and Havlik (1986). A detailed description was given by Remie et al. (1990).

PROCEDURE
Rats are anesthetized with N_2O_2/O_2/halothane. The shaven neck of the animal on the right side is disinfected with chlorhexidine solution. The incision is made just above the right clavicle. Connective and adipose tissue are pushed aside with blunt forceps and the jugular vein is exposed. The external jugular vein is followed and the division into the maxillary vein, the facial and the linguofacial vein identified. The largest vein is chosen and mobilized for a distance of about 5 mm. Small artery forceps are used to clamp the vessel. The vein is then ligated rostral to the clamp with 6-0 silk, and a second ligature is put loosely around the vessel, but not tightened. Using iridectomy scissors, a V-shaped hole is cut in the vein 2 mm rostral from the bifurcation. Prior to its insertion into the vessel, a sterile cannula is connected to a 1 ml syringe filled with a heparinized saline solution. The vessel is dilated by means of a sharp pointed jeweler's forceps, the cannula slit between the legs of the forceps and gently pushed into the vessel until the tip is at the level of the right atrium. Then the forceps is removed, the caudal ligature gently tied, and the rostral ligature used to anchor the cannula to the vessel. The cannula is tunneled to emerge at the top of the head. While the skin in the neck is held firmly, the artery forceps is inserted subcutaneously in caudal direction over a distance of about 3 cm, then turned anti-clockwise in the direction of the incision in the neck. The cannula is grasped with the forceps. Then the forceps is pulled back until the cannula emerges at the crown of the head and closed by a small microvascular clamp. The cannula is slid over the short end of a 20G stainless steel needle bent to a 90° angle. The catheter is flushed with saline and filled with polyethylene/heparin solution. The long end of the L-shaped stainless steel adapter is closed with a piece of heat-sealed PE-tubing and the wounds are closed with sutures.

MODIFICATIONS OF THE METHOD
Hutchaleelaha et al. (1997) described a simple apparatus for serial blood sampling from the external jugular vein which permits simultaneous measurement of locomotor activity in freely moving rats.

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Nicolaidis S, Rowland N, Meile MJ, Marfaing-Jallat P, Pesez A (1974) A flexible technique for long term infusion in unrestrained rats. Pharmacol Biochem Behav 2:131–136
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A.1.3.6.2 Permanent cannulation of the renal vein in rats

PURPOSE AND RATIONALE
A detailed description for permanent cannulation of the renal vein in rats was given by Remie et al. (1990).

PROCEDURE
Rats are anesthetized with N_2O_2/O_2/halothane. After opening the abdominal wall, the intestines are lifted out and laid next to the animal on the right side (as viewed by the surgeon) on gauze moistened with warm saline solution. This provides an excellent view to the vena cava. At its confluence with the vena cava, the right renal vein is stripped of its adipose tissue and the peritoneum is opened. Using small anatomical forceps the peritoneum is detached from the vena cava by making small spreading movements with the forceps just beneath the peritoneum. Subsequently, the vena cava and the renal vein are mobilized for approximately 1.5 cm, to allow for clamping of the vessel. A four or five fine-stitch purse-string is placed in the vessel at the confluence of the vena cava and the right renal vein. Using a Barraquer needle holder and a cotton-wool

stick, the 7-0 silk suture, armed with a BV-1 needle, is guided through the vessel. After each stitch, any bleeding has to be immediately arrested by applying light pressure using a cotton-wool stick. Having completed the suture a single knot is made with the drawstrings. Three microvascular clips are then placed on the vena cava and the renal vein; first the proximal clip on the vena cava, followed by the clip on the renal vein and finally the distal vena cava clip. A small aperture is cut immediately inside the purse-string suture using iridectomy scissors and jeweler's forceps. The cannula, which is filled with a heparinized saline solution, is pushed into the opening as far as possible. Subsequently, the purse-string suture is pulled taut and the clip of the renal vein removed, while pushing the cannula further. The proximal clip on the vena cava is now removed as quickly as possible. The patency of the cannula is checked and the drawstrings of the purse-string suture are used to anchor the cannula. The cannula is laid kink-free in the abdominal cavity and sutured to the internal abdominal cavity near the xiphoid cartilage. The abdomen is closed in two layers and the cannula tunneled to the top of the head. The cannula together with a L-shaped adapter is fixed to the skull.

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A.1.3.6.3 Permanent cannulation of the portal vein in rats

PURPOSE AND RATIONALE

Several techniques have been described for cannulation of the portal vein in rats (Hyun et al. 1967; Pelzmann and Havemeyer 1971; Suzuki et al. 1973; Sable-Amplis and Abadie 1973; Helman et al. 1984). A detailed description for permanent cannulation of the portal vein in rats was given by Remie et al. (1990). After additional application of platinum electrodes around the portal vein in close proximity to the catheter tip, this model can also be used to study the presynaptic regulation of neurotransmitter release from nonadrenergic nerve terminals (Remie and Zaagsma 1986; Remie et al. 1988, 1989).

PROCEDURE

Rats are anesthetized with N_2O_2/O_2/halothane. After opening the abdominal wall, the intestines are lifted out and laid next to the animal on the right side (as viewed by the surgeon) on gauze moistened with warm saline solution. Using a micro needle holder and a cotton-wool stick, a four or five, fine stitch purse-string suture (7-0 silk suture armed with a BV-1 needle) is placed in the wall of the portal vein at the side opposite the gastroduodenal vein. The diameter of the purse-string should be about 1 mm. After the suture has been completed a single knot is made with the drawstrings. The portal vein is clamped with a small curved hemostatic bulldog clamp. Using iridectomy scissors and a pair of jeweler's forceps the center of the purse-string is cut, a cannula filled with heparinized saline is inserted into the vessel and pushed upwards. The purse-string is gently tightened taking care not to obstruct the cannula. The drawstrings of the suture are used to anchor the cannula. The cannula is laid kink-free in the abdominal cavity and sutured to the internal abdominal cavity near the xiphoid cartilage. The abdomen is closed in two layers and the cannula tunneled to the top of the head. The cannula together with a L-shaped adapter is fixed to the skull.

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A.1.3.6.4 Permanent cannulation of the thoracic duct in rats

PURPOSE AND RATIONALE

Collection of lymph is rather difficult and has performed mainly in dogs (Biedl and Offer 1907; Gryaz-

nova 1962, 1963; Vogel 1963). Some techniques have been described for the rat (Bollman et al. 1948; Girardet 1975). Remie et al. (1990) did not obstruct the duct by placing a purse-string suture in the wall of the duct, by which the cannula is secured. The animal's lymph can be collected during the experiment, and after refilling the cannula the lymph flow remains undisturbed.

PROCEDURE

Rats are anesthetized with N_2O_2/O_2/halothane. After opening the abdominal cavity, the intestines are placed in gauze moistened with warm saline and laid to the left of the animal. The suprarenal abdominal artery is located and mobilized by gently tearing the connective tissue. Using blunt dissection technique, the thoracic duct is mobilized along the dorsolateral surface of the aorta. A small three to four fine stitch purse-string suture is placed in the wall of the duct, using a 9-0 Ethilon suture. A hole is cut inside the purse-string with a very fine pair of scissors, while holding the wall with angled jeweler's forceps. The cannula which is filled with heparinized saline solution and is inserted into the duct using anatomical forceps. After the tip of the cannula has been inserted into the thoracic duct, the curved forceps are removed and the total tip is pushed into the duct. The ligature is then closed and some lymph will flow into the cannula. The cannula is secured within the abdominal cavity by attaching it to the abdominal muscle near the xiphoid cartilage with a 7-0 silk suture. Following the closure of the abdominal wall and the tunneling of the cannula to the crown of the head, a L-shaped adapter is placed on the cannula, filled with PVP-solution and closed with a heat-sealed polyethylene cap.

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A.1.3.6.5 Portacaval anastomosis in rats

PURPOSE AND RATIONALE

In 1877 the Russian surgeon Eck reported the achievement of successful portacaval shunts in dogs. Lee and Fischer (1961), Funovics et al. (1975), de Boer et al. (1986), described portacaval shunt in the rat. A detailed description of surgery for portacaval anastomosis in rats was given by van Dongen et al. (1990).

PROCEDURE

Rats are anesthetized with N_2O_2/O_2 in combination with either enflurane, methoxyflurane or isoflurane. After opening the abdominal wall, the intestines are placed left to the animal on gauze moistened with warm saline solution. Proximally and distally to the animal's right renal vein, the vena cava is then stripped of its adipose and connective tissue, and the retroperitoneal cavity is opened. Using anatomical forceps the peritoneum is dissected from the vena cava by making small spreading movements with the forceps just above the vena cava. The portal vein is pulled slightly to the left using straight anatomical forceps and freed from the hepatic artery and the gastroduodenal artery with curved anatomical forceps. Rostral to the celiac artery, the abdominal artery which is covered with peritoneum is freed from its lateral muscle bed over a length of approximately 5 mm providing enough space for placing a small bulldog clamp at a later stage of the operation. Without occlusion a six fine-stitch purse-string is placed in the wall of the vena cava close to its confluence with the right renal vein. Using a Barraquer needle holder and a cotton wool stick, the 7-0 silk suture armed with a BV-1 needle is guided through the vessel. After each stitch, bleeding has to be arrested immediately, by applying light pressure on the area, again using the cotton wool stick. After the suture has been completed, a single knot is made with the drawstrings. The drawstrings should come together at the rostral part of the purse-string. A bulldog clamp, modified to resemble a Satinsky vascular clamp, is then placed on the vena cava.

Before clamping the abdominal aorta rostral to the celiac artery with a small bulldog clamp, a ligature (7-0 silk) is placed around the portal vein as close as possible to the hilus of the liver. Subsequently, the clamp is placed on the aorta and the ligature tightened. A Heifetz clip is then placed transversely onto the portal vein at its confluence with the gastroduodenal vein. The portal vein is cut just distally from the ligature. A prepared button is slipped over the left-hand straight small anatomical forceps, while the right-hand forceps are used to pass the portal vein to the left-hand ana-

tomical forceps. The vein is then grasped and pulled through the button. Subsequently, the button is pushed as close as possible to the Heifetz clip, and clamped to the clip using a Pilling bulldog clamp.

Using small straight, and curved anatomical forceps the portal vein is reversed around the button and fixed with a previously prepared 7-0 silk suture. The Pilling bulldog clamp is then removed and replaced at the end of the Satinsky clamp for reasons of stability. The vena cava is then somewhat elevated, bringing it into closer contact with the portal vein button.

A longitudinal cut is made in the purse-string suture using iridectomy scissors and jeweler's forceps. One drawstring of the suture is clamped with a small hemostat and put under slight tension in a rostral direction. The button manipulated by its grip, is pushed into the vena cava. The purse-string is tightened with the left hand whilst the right hand still holds the button in position. The button is released and two additional knots tied. The Satinsky clamp is removed first followed by the Heifetz clamp and the bulldog clamp on the aorta. After replacing the intestines the abdominal wall is closed in two layers.

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A.1.3.7 Cardiovascular analysis in anesthetized mice

PURPOSE AND RATIONALE

To fully utilize the potential of mouse models with specific gene mutations, it is necessary to study the functional consequences of genetic manipulations in fully intact mice. Lorenz and Robbins (1997) developed and validated a methodology to study cardiovascular parameters in closed-chest mice.

PROCEDURE

Adult mice of either sex weighing 25–35 g are anesthetized by intraperitoneal injection of 50 mg/kg ketamine and 100 mg/kg thiobutabarbital. After the mice are placed on a thermally controlled surgical table with body temperature continually monitored via a rectal probe, a tracheotomy is performed with a short length (<1 cm) of PE-90 tubing. The right femoral artery is then cannulated with polyethylene tubing which is pulled over a flame to a small diameter (~0.4 mm OD). The catheter is advanced ~1 cm, near the level of the aorta, and connected directly to a low-compliance COBE CDXIII fixed-dome pressure transducer for the measurement of arterial blood pressure. The right femoral vein is then cannulated with the same type of small-diameter tubing and connected to a microinjection pump for the infusion of experimental drugs. To assess myocardial performance, the right carotid artery is cannulated with a 2F Millar MIKRO-TIP transducer (Model SPR-407, Millar Instruments, Houston TX). This high-fidelity transducer, which has a tip diameter of ~0.67 mm, has a reported frequency response that is flat up to 10 000 Hz and therefore can be used to accurately monitor the high frequency of the mouse ventricular pulse pressure. During continual monitoring of the blood pressure wave to ascertain the anatomic position of the catheter, the tip of the transducer is carefully advanced through the ascending aorta and into the left ventricle. When the stable waveform of the ventricular pressure profile is achieved, the transducer is anchored in place with 7-0 silk sutures. After completion of the surgery, all wounds are closed with cyanoacrylate to minimize evaporative loss of fluid, and the animals are allowed to stabilize for 30–45 min.

EVALUATION

Blood pressure signals from the COBE transducer and from the Millar transducer are amplified and the output is recorded and analyzed with a MacLab 4/s data acquisition system connected to a Macintosh 7100/80 computer which allows the calculation of the following parameters:

dP/dt	first derivative of the ventricular pressure wave,
MAP	mean arterial pressure,
HR	heart rate,
LVP	systolic and diastolic left ventricular pressure,
LVEDP	left ventricular enddiastolic pressure.

Further indices of ventricular performance can be calculated from *dP/dt*.

MODIFICATIONS OF THE METHOD

Champion et al. (2000) described a **right-heart catheterization technique** for *in vivo* measurement of vascular responses in lungs of intact mice. CD1 mice weighing

25–38 g were anesthetized with thiopentobarbital (85–95 mg/kg i.p.) and ketamine 3 mg/kg i.p.) and were strapped in supine position to a thermoregulated fluoroscopic table. The trachea was cannulated and the animals breathed with room air enriched with 95% O_2/5% CO_2. A femoral artery was cannulated for the measurement of systemic arterial pressure. Heart rate was electronically monitored from the systolic pressure pulses with a tachometer (Grass model 7P44A). The left jugular vein was cannulated for the administration of agonists and antagonists.

For measuring pulmonary arterial pressure, a special single lumen catheter was constructed. The catheter was 145 mm in length and 0.25 mm in outer diameter, with a specially curved tip to facilitate passage through the right heart, main pulmonary artery, and the left or right pulmonary artery. Before the catheter was introduced, the catheter curve was initially straightened with a 0.010-in. straight angioplastic guide wire to facilitate passage from the right jugular vein into the right atrium at the tricuspid valve under fluoroscopic guidance. As the straight wire was removed, the natural curve facilitated entry of the catheter into the right ventricle. A 0.010-in. soft-tip coronary artery guide wire was then inserted, and the catheter was passed over the guide wire into the main pulmonary artery under fluoroscopic guidance. Pressure in the main pulmonary artery was measured with a pressure transducer, and mean pulmonary artery pressure was derived electronically and recorded continuously.

Cardiac output was measured by the thermodilution technique. A known volume (20 µl plus catheter dead space) of 0.9% NaCl solution at 23 °C was injected into the right atrium, and changes in blood temperature were measured at the root of the aorta. A cardiac output computer equipped with a small-animal interface was used. The thermistor microprobe was inserted into the right carotid artery and advanced to the aortic arch, where changes in aortic blood temperature were measured. A catheter placed in the right jugular vein was advanced to the right atrium or main pulmonary artery for rapid bolus injection of saline. The saline solution was injected with a constant-rate syringe to ensure rapid and repeatable injection of the saline indicator solution. Thermodilution curves were recorded on a chart recorder and pulmonary and systemic blood pressure monitored continuously. Catheter placement was verified by postmortem examination.

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A.1.3.8 Blood pressure in anesthetized cats

PURPOSE AND RATIONALE

Cats are the most sensitive species of cardiovascular regulation. They were used extensively for cardiovascular screening. Recently, experiments in dogs are preferred since this species can be bred more easily in homogeneous strains.

PROCEDURE

Adult cats of either sex weighing 2.5 to 4 kg are anesthetized by intraperitoneal injection of 35 mg/kg pentobarbital sodium. Tracheotomy is performed and a tracheal cannula is inserted so that the cat can be mechanically ventilated with room air. A femoral artery and two femoral veins are cannulated for measurement of arterial blood pressure and systemic administration of drugs. The arterial cannula is connected to a Statham model P23Gb transducer. All recordings are made on a polygraph. Heart rate is determined with a Beckman Cardiotach connected to a voltage/pressure-pulse coupler. Rectal temperature is monitored and maintained between 37 °C and 38 °C with a heating pad.

The following drugs are injected i.v. as challenges:

- epinephrine 0.1, 0.3, 0.5 µg/kg,
- norepinephrine 0.1, 0.3, 0.5 µg/kg,
- isoproterenol 0.1, 0.2, 0.4 µg/kg,
- carbachol 0.1, 0.2, 0.5 µg/kg.

At least 5 min are allowed between challenge doses to permit the measured parameters to return to baseline.

Test drugs are injected at various doses followed by injections of the challenging drugs.

EVALUATION

Dose-response curves of challenging drugs are established before and after injections of the test drugs.

CRITICAL ASSESSMENT OF THE METHOD

Blood pressure experiments in anesthetized cats are very valuable as screening techniques for cardiovascular agents. Moreover, potentiation of norepinephrine response has been used as screening procedure for antidepressants with norepinephrine uptake inhibiting activity.

MODIFICATIONS OF THE METHOD

Yardley et al. (1989) studied cardiovascular parameters in spinal cats. The animals were anesthetized with 80 mg/kg intravenously administered α-chloralose. The spinal cord was transected or crushed at the first cervical segment after tetracaine hydrochloride (0.125 mg

in 0.1 ml) had been injected into this region of the cord. Systemic blood pressure was supported at a level sufficient to maintain constricted pupils (mean value 45 ±5 mm Hg) by volume expansion with blood from a donor (10–20 ml) or an infusion of dextran.

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A.1.3.9 Cardiovascular drug challenging experiments in anesthetized dogs

PURPOSE AND RATIONALE

Sympathomimetic and cholinomimetic compounds as well as angiotensin II and carotid occlusion exert characteristic responses in blood pressure of anesthetized dogs. Antagonism or potentiation of these responses allow to characterize the cardiovascular activity of a new compound.

PROCEDURE

Adult Beagle dogs of either sex weighing between 8 and 15 kg are anesthetized with 15 mg/kg sodium thiopental, 200 mg/kg sodium barbital and 75 mg/kg sodium pentobarbital. Additional doses of sodium pentobarbital are given as needed. The dogs are intubated with a cuffed endotracheal tube and placed on a Harvard respirator (20 ml/kg, 10–15 cycles/min). A femoral vein and artery are cannulated using polyethylene tubing for drug administration and determination of arterial blood pressure, respectively. The animals are bilaterally vagotomized.

The arterial cannula is connected to a Statham model P23Gb transducer. All recordings are made on a polygraph. Heart rate is determined with a Beckman Cardiotach connected to a voltage/pressure-pulse coupler.

Drug challenges: One of the following combinations of drugs is administered i.v. to the dogs. The challenges are given in a fixed order: at least twice prior to test drug administration to insure consistent responses and again starting 15 min post test drug. Epinephrine and norepinephrine (1 µg/kg), isoproterenol (0.25 µg/kg), carbachol (0.25 µg/kg), tyramine (100 µg/kg) are used; bilateral carotid occlusion (45 s), phenylephrine (10 µg/kg), isoproterenol (0.25 µg/kg) angiotensin II (0.2 µg/kg) and carbachol (0.25 µg/kg) for cardiovascular drugs. At least 5 min are allowed between challenge doses to permit the measured parameters to return to baseline. Challenge drug doses are sometimes varied to keep the mean arterial pressure within the following limits: epinephrine (+30 to +60 mm Hg), norepinephrine (–30 to +70 mm Hg), tyramine (+30 to +70 mm Hg), isoproterenol (–30 to –50 mm Hg), carbachol (–30 to –50 mm Hg), phenylephrine (–30 to +70 mm Hg), angiotensin II (+30 to +50 mm Hg), and bilateral carotid occlusion (+30 to +70 mm Hg).

EVALUATION

The recordings are studied to detect any changes in the arterial pressure response to the challenge drug before and after test-drug administration and to observe any changes in blood pressure and heart rate. Results are expressed as the percentage change from the predrug response.

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A.1.3.10 Hemodynamic analysis in anaesthetized dogs

PURPOSE AND RATIONALE

The hemodynamic effects of compounds supposed to affect the cardiovascular system are evaluated by measuring preload and afterload of the heart, contractility,

heart rate, cardiac output and peripheral or coronary flow. To measure these cardiovascular parameters accurately, the use of larger animals such as dogs or pigs is necessary.

This experimental model allows the classification of test drugs according to their action as having:

- positive inotropic effects
- negative inotropic effects (Ca^{2+}-antagonist, anti-arrhythmic?)
- hypertensive effects
- hypotensive effects
- coronary-dilating effects
- β-blocking effects
- α-blocking effects
- anti-anginal effects
- peripheral-vasodilating effects

PROCEDURE

Male or female inbred Beagle or Labrador-Harrier dogs weighing between 15 and 25 kg are used. They are anesthetized with a bolus injection of 35–40 mg/kg pentobarbital, and continued with an infusion of 4–6 mg/kg/h. A catheter is placed into the cephalic vein for intravenous injections. Another catheter is placed into the duodenum for enteral administration. Respiration is maintained with room air through a tracheal tube using a positive pressure respirator, e.g., Bird-Mark-7-respirator. Blood gas analyses are performed at regular time intervals. Oxygen is supplied via the respirator as needed.

Preparation for hemodynamic measurements

Blood pressure is recorded through a cannula inserted into the left femoral artery and connected to a Statham pressure transducer (Statham P 23 DB).

For determination of LVP, a Millar microtip catheter (type PC 350) is inserted via the left common carotid artery into the left ventricle. LVEDP is measured on a high-sensitivity scale. From the pressure curve, dp/dt_{max} is differentiated and heart rate is counted. The LVP-signal also triggers a cardiotachometer.

Cardiac output, pulmonary artery pressure (PAP) and stroke volume are measured by a thermodilution technique using a Cardiac Output Computer (Gould/Statham SP 1245) and a balloon-tip triple lumen catheter (Gould SP 5105, 5F) with the thermistor positioned in the pulmonary artery via the jugular vein.

Myocardial oxygen consumption (MVO_2) is calculated as pressure-work-index according to Rooke and Feigl (1982).

Femoral blood flow and coronary flow are measured with electromagnetic flow probes attached to the femoral artery and the circumflex branch of the left coronary artery (LCX), respectively.

Experimental course

When stable hemodynamic conditions and blood gas values of $pO_2 > 100$ mm Hg and $pCO_2 < 35$ mm Hg are achieved for at least 20 min (control values), the test substance is administered through a catheter inserted into a cephalic vein in doses of 0.1, 0.3, 1.0, and 3.0 mg/kg or into the duodenum in doses of 0.3, 1.0, 3.0, and 10.0 mg/kg.

All parameters are recorded continuously during the whole experiment.

Characteristics

- blood pressure
  - systolic, BPs
  - diastolic, BPd
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure, LVEDP
- maximal rate of pressure rise, *dp*/*dt* max
- heart rate, HR
- peripheral blood flow in A. femoralis, PF
- blood pressure A. pulmonalis, PAP
- coronary flow, CF
- cardiac output, CO
- stroke volume, SV
- total peripheral resistance, TPR
- left ventricular stroke work, LVSW
- left ventricular minute work, LVMW
- left ventricular myocardial oxygen consumption, MVO_2

CALCULATION OF RESULTS AND EVALUATION

Besides the different directly measured hemodynamic parameters, the following data are calculated according to the respective formulae

- stroke volume [ml/beat],

$$SV = \frac{CO}{HR}$$

- total peripheral resistance [dyn s/cm^5],

$$TPR = \frac{BPm}{CO} \times 79.9$$

- left ventricle stroke work [J/beat],

$$LVSW = (BPm - LVEDP) \times SV \times 0.333 \times 10^{-3}$$

- left ventricular minute work [J/min],

$$LVMW = LVSW \times HR$$

- left ventricular myocardial oxygen consumption [ml O_2/min/100 g],

$$MVO_2 = K_1(BPs \times HR) + K_2 \times \frac{(0.8BPs + 0.2BPd) \times HR \times SV}{BW} + 1.43$$

K_1 = 4.08×10^{-4}
K_2 = 3.25×10^{-4}
BPs = systolic blood pressure [mm Hg]
BPd = diastolic blood pressure [mm Hg]
BPm = mean blood pressure [mm Hg]
HR = heart rate [beats/min]
CO = cardiac output [ml/min]
SV = stroke volume [ml/beat]
LVEDP = left ventricular enddiastolic pressure [mm Hg]
BW = body weight [kg]

Changes in parameters measured after drug administration are compared to control values obtained during the 20 min pre-drug period.

Results are presented as mean ±SEM with $n > 3$.

Statistical significance is assessed by means of the paired *t*-test.

MODIFICATIONS OF THE METHOD

The effect of drugs on the carotid artery occlusion effect can be studied in anesthetized dogs. The occlusion of right and left common carotid arteries is performed by squeezing them between a polyethylene tubing and a twine which is passed inside the tubing and around the carotid artery. An occlusion of the carotid arteries for 30 s causes an increase of systolic blood pressure by 40–50 mm Hg. Inhibition of this effect by drugs is tested.

Studies in anesthetized dogs can be used to determine the influence of cardiotonic drugs on propranolol induced cardiac insufficiency (Rajagopalan et al. 1993)

Instead of dogs, **pigs (German landrace)** weighing between 20–35 kg can be used. They are pretreated with ketamine 500 mg/5 ml i.m., methomidate hydrochloride 200 mg/4 ml i.p., xylazine 60 mg/3 ml i.m., and anaesthetized with 15–20 mg/kg pentobarbital sodium, followed by continuous infusion of 12 mg/kg/h. The parameters are evaluated similarly to the experiments in dogs.

Measurement of cardiac output by the thermodilution method in **rats** was described by Richardson et al. (1962) and Müller and Mannesmann (1981).

Thermodilution methods were used by Rosas et al. (1964) in anesthetized rats, by Carbonell et al. (1985) and by Salyers et al. (1988) in conscious rats to determine hemodynamic parameters.

Oxygen pressure, carbon dioxide pressure and pH in coronary venous and common carotid arterial blood of anesthetized dogs has been measured using a blood gas analyzer (Aisaka et al. 1988).

Acute ischemic left ventricular failure can be induced in anesthetized dogs by repeated injections of plastic microspheres into the left coronary artery (Smiseth and Mjøs 1982; Sweet et al. 1984; Schölkens et al. 1986). A coronary catheter was introduced through the right femoral artery and advanced under fluoroscopy to the left coronary ostium, guided by injection of small amounts of contrast medium. After reaching baseline values, acute left ventricular failure was induced by subsequent intra-coronary injections of plastic microspheres (52.9 ±2.48 µm non-radioactive tracer microspheres). The microspheres were suspended in saline with a drop of Tween 80 and sonified before use, 1 mg microspheres/1 ml saline corresponding to approximately 12 000 microspheres. (13–16 injections of microspheres or 3.4–5.0 mg/kg). Microspheres were injected every 5 min for 70–90 min. Each microsphere injection effected an immediate and stepwise increase in LVEDP. With this procedure, LVEDP can be increased to a desired level in a very controlled manner. In the 30 min following embolization, LVEDP continued to increase by approximately 5 mm Hg. Animal with arrhythmias had to be excluded from the study. Thirty min after the end of embolization, when hemodynamic parameters had stabilized, drug administrations were started.

Valdes-Cruz et al. (1984) developed an **open-chest preparation in dogs** to validate the accuracy of a two-dimensional Doppler echocardiographic method for estimating pressure drops across discrete stenotic obstructions.

In order to assess the potential of a single breath technique (using freon-22) as an effective way to estimate cardiac output non-invasively, Franks et al. (1990) measured simultaneously with the single breath technique the aortic flow using an electromagnetic flowmeter in anesthetized dogs.

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A.1.3.11 Hemodynamic measurements in conscious dogs

PURPOSE AND RATIONALE

The potency of a cardiovascular drug depends on the direct effects at the cellular level and on the response of the cardiovascular control mechanisms. The latter are often markedly influenced by anesthesia. The chronically instrumented conscious dog with renal hypertension is therefore a more realistic test model to evaluate the effects of antihypertensive, anti-anginal and cardiotonic compounds. The test is used to evaluate hemodynamic drug effects in conscious dogs, an experimental model with chronic arterial and ventricular catheterization and renal artery constriction.

PROCEDURE

Male or female Labrador-Harrier dogs weighing 15–25 kg are used. They are anaesthetized with 1 mg/kg xylazine i.m., followed by 1 mg/kg xylazine i.v. and 18 mg/kg pentobarbital sodium i.v. For chronic instrumentation and induction of renal hypertension, fluid-filled catheters are implanted into the abdominal aorta and into the left ventricle. The catheters are tunneled subcutaneously and exteriorized on the nape of the neck dorsally. Renal hypertension is induced by placing silastic constrictors around both renal arteries. Hemodynamic measurements are performed after a two-week recovery period or later.

To familiarize the dogs to the test surroundings, they are brought into the laboratory 2–3 times before the start of the study. Thus, drug testing is possible without sedation. During the experiment the animal rests quietly on a laboratory table.

Experimental protocol

Hemodynamic measurements are performed by connecting the two implanted catheters to Statham pressure transducers. Pressure signals, electronically differentiated LVP *dp/dt* max and heart rate are recorded with a polygraph.

After reaching stable hemodynamic conditions for at least 20 min (control baseline values), the test compound is administered either orally in a gelatin capsule or by intravenous injection into the cephalic vein.

Hemodynamic parameters are recorded continuously starting 30 min before to 120 min after drug administration, and thereafter at 1 h intervals until 6 h after dosage.

EVALUATION

The following parameters are monitored:

- systolic blood pressure [mm Hg]
- diastolic blood pressure [mm Hg]
- left ventricular enddiastolic blood pressure, LVEDP [mm Hg]
- left ventricular pressure at *dp/dt* max [mm Hg/s]
- heart rate [beats/min]

Mean values ±SEM are calculated with $n > 3$ as differences to pre-drug control values.

MODIFICATIONS OF THE METHOD

Mann et al. (1987) described a simple procedure for direct blood pressure measurement in conscious dogs using the Vascular-Access-Port™, consisting of a 33 × 13 mm reservoir body affixed to a silicon rubber catheter.

Müller-Schweinitzer (1984) described a method for the assessment of vasoconstrictor agents by recording venous compliance in the conscious dog. Changes in the diameter of the canine saphenous vein, produced by inflation to 45 mm Hg of a sphygmomanometer cuff placed on the upper hind leg, were recorded.

Hintze and Vatner (1983) compared the effects of nifedipine and nitroglycerin in conscious dogs, instrumented for instantaneous and continuous measurements of coronary arterial and left ventricular diameters with an ultrasonic dimension gauge, arterial and left ventricular pressure with implanted miniature gauges, and coronary blood flow with an electromagnetic flowmeter or a Doppler ultrasonic flowmeter.

Shimshak et al. (1986) studied the recovery of regional myocardial contractile function after a 10 min coronary artery occlusion in chronically instrumented conscious dogs.

Wright et al. (1987) described a minimally invasive technique which allows assessment of histamine H_1-receptor antagonist activity in conscious dogs based on the inhibition of tachycardia caused by intravenous administration of the H_1-receptor agonist, 2-pyridylethylamine.

Hashimoto et al. (1991) studied the coronary effects of nicorandil in comparison with nitroglycerin in chronic conscious dogs instrumented with ultrasonic crystals and electromagnetic flowmeters in the circumflex coronary artery.

Hartman and Warltier (1990) described a model of multivessel coronary artery disease using conscious, chronically instrumented dogs. A hydraulic occluder and Ameroid constrictor were implanted around the left anterior descending and the left circumflex coronary arteries. Pairs of piezoelectric crystals were implanted within the subendocardium of the left anterior descending and the left circumflex coronary artery perfusion territories to measure regional contractile function. A catheter was placed in the left atrial appendage for injection of radioactive microspheres to measure regional myocardial perfusion.

Hof et al. (1990) used the Doppler method for measuring cardiac output in **conscious rabbits**.

Grohs et al. (1993) simultaneously assessed cardiac output with pulsed Doppler and electromagnetic flowmeters during cardiac stimulation.

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A.1.3.12 Hemodynamic studies in monkeys

PURPOSE AND RATIONALE

Prior to studies in human beings, studies of cardiovascular effects of new drugs in monkeys are necessary in some instances.

PROCEDURE

Rhesus monkeys of either sex, weighing between 5 and 8 kg are anesthetized with 20 mg/kg ketamine hydrochloride followed by 50 mg/kg pentobarbital-Na given slowly i.v. A small side-branch of the femoral or radial artery is surgically exposed and cannulated for blood pressure recordings using a blood pressure transducer (P23 ID). Heart rate is determined from a conventional ECG lead by a biotachometer. Compounds are administered either intravenously or via a gastric fibroscope, e.g., Olympus XP 10, into the duodenum

under visual control. The cardiovascular parameters are registered for a pretest period of 30 min and then during 60 min after intravenous administration or 2 h after intragastric administration of the test drug. Three to 6 animals are used for evaluation.

EVALUATION

Mean values ±SD are calculated for the pretest period and for the cardiovascular effects every min for 5 min after i.v. administration and then every 5 min. After intragastric administration the values are registered every 5 min up to 30 min and then every 10 min. The values after administration of the test compound are compared statistically with the pretest values using the Student's *t*-test.

MODIFICATIONS OF THE METHOD

Lacour et al. (1993) studied cardiovascular parameters in conscious **cynomolgus monkeys** (*Macaca fascicularis*). A silicone catheter (internal and external diameter 0.64 and 1.19 mm, respectively) was implanted under aseptic conditions into the thoracic aorta via a carotid artery after the monkeys had been anesthetized with 40 mg/kg ketamine and 0.5 mg/kg acepromazine intramuscularly. The vascular catheter (filled with an aqueous solution of 40% polyvinylpyrolidone and 20% heparin) was inserted into a carotid artery. A patch of silicone was sewn around the artery to maintain the catheter in position, the latter being routed subcutaneously and exteriorized at the top of the head into a stainless steel connector. This connector was fixed to the skull with screws and dental cement, and sealed with a plug to protect the catheter from damage. The monkeys were permitted a 3-week minimum recovery period. Before the experiment was performed the monkeys were placed in a primate-restraining chair on several occasions, of gradually increasing duration, for experiment acclimatization.

Pulsatile arterial pressure was recorded by connecting the arterial catheter to a polygraph via a Statham P23Id pressure transducer. Mean arterial pressure and heart rate were derived from the pulse pressure signal and recorded. A catheter was inserted acutely into a saphenous vein for administration of compounds.

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A.1.3.13 Measurement of cardiac output and regional blood flow with microspheres

PURPOSE AND RATIONALE

The microsphere technique allows the measurement of cardiac output and regional blood flow. Using different radionuclides, repeated determinations are possible. The method is applicable not only for dogs, cats, and minipigs (Hof et al. 1980) but also for rats (McDevitt and Nies 1976; Bonnacrossi et al. 1978; Ishise et al. 1980; Stanek et al. 1985) using microspheres of appropriate size.

PROCEDURE

Male Sprague-Dawley rats weighing 265–375 g are anesthetized with 35 mg/kg i.p. pentobarbital. The right carotid and right femoral arteries are cannulated. Using pressure monitoring, a carotid cannula is manipulated into the left ventricle. Carbonized micro-spheres (15 ±5 μ diameter) labelled with ^{85}Sr are drawn into a glass injection chamber and suspended in 0.3 ml 6% dextran so that each chamber contains 60 000 to 80 000 microspheres. The radioactivity in each chamber is determined by gamma scintillation counting before and after microsphere injection, the difference being the amount of radioactivity injected. The microspheres are injected into the left ventricle in a total volume of 0.8 ml 6% dextran over 20 s. Simultaneously, arterial blood from the femoral artery is withdrawn at 0.8 ml/min for 90 s with a syringe withdrawal pump.

EVALUATION

This reference blood sample is used to calculate the cardiac output by the formula:

$$\text{cardiac output} = \text{counts injected} \times \frac{\text{reference sample withdrawal rate}}{\text{reference sample counts}}$$

After obtaining the reference sample, the animals are sacrificed with pentobarbital and the organs dissected, placed in counting vials, and counted for 5 min. Regional distribution of the cardiac output is calculated by comparing the radioactivity in each organ with the total injected radioactivity. Organ flow is determined by multiplying the cardiac output by the fractional distribution of the cardiac output to the organ.

CRITICAL ASSESSMENT OF THE METHOD

Problems associated with the microsphere technique in rats are the hemodynamic effects of the solutions

used to inject the microspheres and the effects of blood withdrawal after repeated determinations (Stanek et al. 1985).

MODIFICATIONS OF THE METHOD

For repeated determinations, other nuclides have been used, such as ^{46}Sc, ^{51}Cr, ^{141}Ce, ^{125}I (Hof et al. 1980).

Kovách et al. (1992) used up to 5 radiolabelled microspheres (^{57}Co, ^{113}Sn, ^{85}Sr, ^{95}Nb and ^{46}Sc) for measurement of regional cerebral blood flow in cats.

Faraci and Heistad (1992) measured blood flow with radioactive microspheres (15 µ diameter) labeled with ^{46}Sc, ^{95}Nb, ^{153}Gd, ^{85}Sr, and ^{141}Ce in anesthetized rabbits.

Grover et al. (1990), Gross et al. (1992) measured myocardial blood flow in dogs with the radioactive microsphere technique.

Kowallik et al. (1991) measured regional myocardial blood flow with multiple colored microspheres. The method yielded values very similar to those obtained with radioactive microspheres.

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A.1.3.14 Carotid artery loop technique

PURPOSE AND RATIONALE

The carotid loop method, originally described by van Leersum (1911) for rabbits has been used by several authors (e.g., Child and Glenn 1938; Valli et al. 1967; O'Brien et al. 1971; Meyer et al. 1989) for measurement of blood pressure or blood sampling in conscious dogs and sheep (Lagutchik et al. 1992).

PROCEDURE

Male or female inbred Beagle or Labrador-Harrier dogs weighing between 15 and 25 kg are used. They are anesthetized with a bolus injection of 35–40 mg/kg pentobarbital, continued with an infusion of 4–6 mg/kg/h. The animal is placed on a heated operating table. The skin on the ventral side of the neck is carefully shaved and disinfected. The course of the carotid artery is outlined by palpation along the tracheal border. About 2 cm of skin is taken on each side marking the width of the flap. The medial incision is made slightly above the thyroid cartilage and is extended caudal to a point about 1 cm lateral and 1 cm above the manubrium sterni. The lateral incision again lies about 2 cm from the line of the carotid artery and parallel to it. The lateral incision is only half as long as the medial one. The incisions are made down to the subcutaneous tissue over the platysma muscle. Between the skin and the muscle, the flap is undermined. All bleeding points are carefully clamped and tied.

The subcutaneous tissue, the platysma myoides muscle and the anterior fascia of the neck are incised in the course of the midline incision down to the plane of cleavage between the sternohyoid and sternomastoid muscle. By blunt dissection, these muscles are separated, disclosing at their depth the neurovascular bundle over which lies the internal jugular vein. The floor of the space so isolated is formed by the longus capitis muscle. By careful dissection, these muscles are separated at least 1 cm above and below the limits of the incision in the skin. The superior thyroid artery marks the uppermost portion of the carotid artery suitable for exteriorization. The plane of cleavage is followed caudal to the origin of the sternocleidomastoid muscle at the manubrium sterni. Throughout the limits of the incision the artery is dissected free from the internal jugular vein and then from the vagus nerve.

The first step in the exteriorization of the artery is the re-approximation of the muscle borders beneath the vessel by mattress sutures. In order to prevent tension on the completed loop due to contraction of the sternomastoid and sternohyoid muscle, it is important to re-approximate these muscles throughout their course. Sutures are placed at the edges of skin. The tubular flap of skin is then approximated loosely around the carotid artery. It is essential that the skin flap fits loosely around the artery. A continuous suture of fine silk is started at the place where the vessel emerges from the muscle borders. The suture is so placed as to include the artery in a sling of skin which isolates the vessel from the line of suture of the under-side of the completed loop. Finally, the proximal and distal quarters of the flap are closed with sutures, while the skin tube is closed with a continuous suture. Antibiotics are given locally and systemically.

One thickness of gauze is placed beneath the loop and along each border a strip of gauze in order to relieve the loop from the pressure caused by the reminder of dressings. Around the neck is wrapped a gauze bandage several turns of which have passed behind the forelimbs in order to prevent the dressing from riding upwards on the animal's neck. Over this is placed a plaster roll protecting the loop from the animal's efforts of scratching. The dressings are changed on the fifth and seventh day when the sutures can be removed.

Blood pressure measurements can be made according to Riva-Rocci's principle by placing a inflatable cuff around the loop.

CRITICAL ASSESSMENT OF THE METHOD

The carotid artery loop method needs some surgical experience and very meticulous care-taking of the animals.

MODIFICATIONS OR THE METHOD

Lewis et al. (1980) placed a CO_2 sensor using mass spectrometry and its through flow cuvette in a common carotid artery-to-jugular vein loop in anesthetized cats.

Meyer et al. (1989a,b) studied pulmonary gas exchange in panting dogs with an exteriorized carotid artery loop.

Kaczmarczyk et al. (1979) used conscious, chronically instrumented dogs with electric flow probes around the left renal artery and a carotid loop to study postprandial volume regulation.

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A.1.3.15 Measurement of heart dimensions in anesthetized dogs

PURPOSE AND RATIONALE

The measurement of the heart dimensions allows to localize the effect of a drug on the activity of the heart. An ultrasonic technique is used for continuous measurement of left ventricular dimensions. Compounds are tested with potential anti-anginal activity due to the reduction of left ventricular diameter. The test is used to evaluate the influence of drugs on left ventricular external and internal diameter in anesthetized dogs.

PROCEDURE

Male or female Beagle or Labrador-Harrier dogs weighing 15–25 kg are used for the test. The dog is anesthetized by intravenous injection of 35–40 mg/kg pentobarbital sodium followed by subcutaneous injection of 2 mg/kg morphine. Respiration is maintained through a tracheal tube with N_2O/O_2 (3:1) using a positive pressure respirator.

Implantation of ultrasonic transducers

Ultrasonic transducers are constructed and implanted as described by Stinson et al. (1974).

To measure left ventricular external diameter (LVED), two ultrasonic transducers are fixed to the left ventricular wall. One crystal is sutured to the posterior wall within the rectangular area formed by the left circumflex coronary artery and the left posterior descending artery. The other one is placed near the first diagonal branch of the left anterior descending coronary artery. Exact positioning is assured with an oscilloscope.

To measure left ventricular internal diameter (LVID), the transducers are placed in the same anatomical area as for the epicardial crystals. However, they are pushed through the wall of the left ventricle through stab

wound incisions. The crystals are positioned across the greatest transverse diameter of the left ventricle, one on the anterior and the other on the posterior endocardial wall.

Bleeding during the implantation procedure is controlled by umbilical tapes around the cranial and caudal veins and by purse string sutures at the implantation sites. The pericardial incision and the chest is closed by sutures and the transducer wires are connected to the recording equipment.

In each dog, either LVED or LVID is measured together with the other hemodynamic parameters.

Preparation for hemodynamic measurements

Blood pressure is recorded through a cannulated femoral artery by a pressure transducer (Statham P 23 DB).

For determination of LVP, a Millar microtip catheter (type PC 350) is inserted via the left A. carotis communis. LVEDP is measured on a high-sensitivity scale. From the pressure curve, *dp*/*dt* max is differentiated and heart rate is calculated.

Hemodynamic parameters are recorded continuously during the whole experiment.

Experimental course

When stable hemodynamic conditions are achieved for at least 30 min (control values), the test substance is administered by intravenous or intraduodenal injection.

Readings are taken at times 0, 15, 30, 45, 60, 75, 90 and 120 after drug administration. Left ventricular dimensions are measured at the end of the diastole and systole.

Characteristics:

- blood pressure
  - – systolic blood pressure
  - – diastolic blood pressure
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure
- left ventricular contractility, dp/dt
- heart rate, HR
- left ventricular external diameter, LVED
- left ventricular internal diameter, LVID

EVALUATION

Hemodynamic parameters, LVED and LVID [mm] are determined.

Changes in parameters after drug administration are compared to control values obtained during the 30 min pre-drug period.

Statistical significance is assessed by means of the paired *t*-test.

Since a change in the diameter of the left ventricle is a reasonable accurate index of left ventricular volume, a reduction of LVED or LVID with no change in *dp*/*dt* and HR can be considered as a strong indicator for "venous pooling" and thus an anti-anginal activity of a compound.

Scores are allotted relative to the efficacy of standard compounds assessing the intensity as well as the duration of the effect.

Standard data:

		LVED [mm]		LVID [mm]	
Nitroglycerin	0.005 mg/kg, i.v.	–0.9	20 min	–1.2	30 min
Isosorbide-dinitrate	0.1 mg/kg, i.v.			–0.6	120 min
Molsidomine	0.2 mg/kg, i.v.	–2.1	>60 min	–1.4	>120 min
Nifedipine	0.1 mg/kg, i.v.			+1.2	120 min

MODIFICATIONS OF THE METHOD

Novosel et al. (1992) measured the dimensions of the right ventricle with microsonometry in anesthetized rabbits.

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A.1.3.16 Telemetric monitoring of cardiovascular parameters in rats

PURPOSE AND RATIONALE

Radiotelemetry allows the recording of cardiovascular parameters in conscious, free-moving animals. Several authors (Brockway et al. 1991; Mattes and Lemmer

1991; Guiol et al. 1992; Morimoto et al. 1992; Basil et al. 1993; Brockway and Hassler 1993; Lemmer et al. 1993, 1994, 1995; Calhoun et al. 1994; Diamant et al. 1993; Kramer et al. 1993a, 1995; Griffin et al. 1994; Kuwahara et al. 1994; Sato et al. 1994, 1995; van den Buuse et al. 1994; Kinter 1996; Becker et al. 1997; Witte et al. 1998) used commercially available systems with some modifications to study the circadian rhythm of blood pressure and the influence of drugs on heart rate, blood pressure and motility in rats.

PROCEDURE

The telemetry and data acquisition system (e.g., Data Sciences International, Inc., St Paul MN) consists of four parts:

1. the implantable transmitter, which measures the pressure. This device contains a highly stable, ion-implant, semiconductor, strain-gauge sensor and battery-powered electronics to process the information from the pressure sensor and to telemeter it from within the animal. Arterial pressure is transmitted to the sensor via a 0.7-mm diameter, fluid-filled catheter;
2. the receiver which detects the signal from the implanted transmitter and converts it to a form readable by computer;
3. the pressure reference module, which measures atmospheric pressure to allow for the telemetered absolute pressure to be converted to a gauge pressure;
4. the data acquisition software, which accepts data from the reference module and the receivers, filters corrupt samples from the incoming data stream, converts the telemetered pressure to millimeters of mercury, subtracts atmospheric pressure from the telemetered pressure, and stores the data for retrieval, plotting, and analysis.

Under pentobarbital anesthesia, the telemetry transmitter is implanted into rats. The descending aorta is exposed between the renal arteries. A vascular clamp is made by putting two surgical threads on the proximal and distal part of the artery. The catheter tip is inserted through an incision in the vessel. A drop of cyanoacrylate glue is applied to the dried entry point. The transmitter is sutured to the abdominal musculature.

EVALUATION

Data from individual animals are recorded over long periods of time which allow the investigator to follow the circadian rhythm under several experimental conditions.

MODIFICATIONS OF THE METHOD

Hess et al. (1996) monitored pulmonary arterial pressure in freely moving **rats** by inserting the sensing catheter of a telemetric system through a small hole and pushing it into the pulmonary artery.

Further cardiovascular studies in rats using the telemetric system were reported by Sgoifo et al. (1998), Webb et al. (1998).

Kramer et al. (1993b) used telemetry to record electrocardiogram and heart rate in freely moving **mice**.

Carlson and Wyss (2000) used small telemetry probes for long term recording of arterial pressure and heart rate in mice after implantation to the carotid artery or the abdominal aorta.

DePasquale et al. (1994) used radio-telemetry to monitor cardiovascular function in conscious **guinea pigs**.

Telemetric ECG recordings in **cardiomyopathic hamsters** were reported by Desjardins et al. (1996).

Van den Buuse and Malpas (1997) studied cardiovascular parameters in **rabbits** by radio-telemetry.

Astley et al. (1991), Smith et al. (1993) used telemetric systems to monitor cardiovascular responses in **baboons**.

Schnell and Wood (1993) measured blood pressure and heart rate by telemetry in conscious, unrestrained **marmosets**.

An ultrasonic blood flowmeter telemetry system for **cats** and rabbits has been described by Yonezawa et al. (1989, 1992).

Telemetry was used by Symons et al. (1992) to monitor the severity of events representing myocardial dysfunction in **miniswine**.

Savory and Kostal (1997) applied the telemetric system for chronic measurement of cardiovascular and other parameters in **chicken**.

Radiotelemetry has also been used for other pharmacological experiments, such as field potential analysis by radioelectroencephalography (see Sect. E.1.2.6), step through passive avoidance (see Sect. F.3.1.2), shock-prod burying test in rats (see Sect. E.2.5.3), measurement of body temperature (see Sect. H.4.0.2) and motility in rats and mice (Clement et al. 1989; Guillet et al. 1990; Diamant et al. 1993; van den Buuse 1994).

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A.1.3.17 Cardiovascular effects after intracerebroventricular administration

PURPOSE AND RATIONALE

Several drugs, like α_2-adrenergic agonists, act primarily at central sites. Their effects can be most clearly demonstrated after injection into the cerebroventricular system. The first experiments have been performed in cats. The method has been adapted to rats.

PROCEDURE

Rats of either sex weighing 250–350 g are anesthetized with 100 mg/kg hexobarbital i.p. The scalp is cut in a sagittal line. With a dental drill a hole of 1–1.5 mm diameter is drilled through the cranial bone 1 mm lateral and 2 mm caudal of the bregma. A PVC-catheter is introduced perpendicular to the bone to a depth of 3 mm in order to reach the lateral cerebral ventricle. The catheter is fixed with dental cement and the wound closed. Test substances are administered through the catheter. To measure blood pressure one catheter is placed in one carotid artery and connected to a Statham transducer. Blood pressure and heart rate are recorded on a polygraph over a period of at least 30 min. For long acting drugs registration periods up to 2 h are necessary. After the experiment, the animal is sacrificed and the brain removed to confirm the site of injection.

EVALUATION

Systolic and diastolic blood pressure as well as heart rate after intracerebroventricular injection are expressed as percentage of pretreatment values. The response is compared with the standard clonidine which is effective in doses of 4–60 µg.

MODIFICATIONS OF THE METHOD

Based on the work of Feldberg et al. (1954) and Hayden et al. (1966), Mastrianni et al. (1986) developed an intracerebroventricular perfusion system for the study of centrally acting antihypertensive drugs in the rat. The antihypertensive effect of clonidine could be observed over several hours.

Methods used to detect central hypotensive activity of drugs have been reviewed by Timmermans (1984).

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A.1.3.18 Influence on orthostatic hypotension

PURPOSE AND RATIONALE

Orthostatic hypotension with dizziness up to unconsciousness is a syndrome occurring in many human individuals. Moreover, several drugs are known to cause orthostatic hypotension. In several animal species, such as rabbit, cat and dog, this syndrome can be evoked by changing the usual horizontal position into a vertical position with the head upwards using a tilting table.

PROCEDURE

Cats of either sex weighing 2.0–3.0 kg are temporarily anesthetized with ether. Anesthesia is maintained by intravenous injection of 70 mg/kg chloralose. The animal is fixed with its legs on a heated operating table which can be tilted by 90 degrees. The carotid artery is cannulated for measuring blood pressure through a Statham P 23 Db transducer on a 6 channel Hellige recorder. The femoral vein is cannulated for injection of the test compound. After the blood pressure is stabilized for 30 min, the animal is quickly tilted to a vertical position for 1 minute. Due to the change of position and gravitational force, there is a rapid fall in blood pressure which recovers as soon as the animal is restored to its original position. After taking the control reading, the test compound is administered intravenously and the same procedure is repeated. The fall in blood pressure is recorded.

EVALUATION

A significant increase in postural hypotension with respect to the control would indicate that the test compound may produce orthostatic hypotension in human. Moreover, some compounds, like sympathomimetics, can reduce or prevent postural hypotension.

MODIFICATIONS OF THE METHOD

Sponer et al. (1981) described a method for evaluating postural hypotension in conscious **rabbits** placed on a tilting table whereby blood pressure was measured from the central artery of the ear.

Humphrey and McCall (1982) described a model for predicting orthostatic hypotension during acute and chronic antihypertensive drug therapy in **rats** anesthetized with chloralose, urethane and pentobarbital using a heated tilting table.

Lee et al. (1982) evaluated postural hypotension induced by drugs in conscious restrained normotensive rats. Blood pressure was recorded after cannulation of the femoral artery under light ether anesthesia. A special tilting table was build for simultaneous studies in four rats.

Baum et al. (1981) studied antihypertensive and orthostatic responses to drugs in conscious **dogs**. A catheter was placed in the subclavian artery for measurement of blood pressure and exteriorized at the back of the neck some days prior to the experiment. The animals were placed into a sling and tilted to the 90° upright position for periods of 60 s. every hour by lifting their forelimbs. Blood pressure response before and after treatment with test drugs was measured.

A none human **primate** model for evaluating the potential of antihypertensive drugs to cause orthostatic hypotension was described by Pals and Orley (1983). Polyvinyl catheters were implanted in the abdominal aorta and the vena cava via an external iliac artery and vein to **cynomolgus monkeys** during ketamine anesthesia. The catheters were routed subcutaneously from the groin area to the top of the head and exteriorized. After recovery the animals were placed in restraining chairs allowing the change from vertical to horizontal position.

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A.1.3.19 Bezold-Jarisch reflex

PURPOSE AND RATIONALE

The circulatory collapse after intravenous injection of veratrine has been first described in cats and is known as BEZOLD-JARISCH-reflex (Bezold and Hirt 1867; Jarisch and Richter 1939a,b, Jarisch 1940). Fleckenstein et al. (1950) recommended this as a suitable animal model of shock.

The original observation was a triphasic blood pressure response in cats or dogs characterized by a short lasting fall in blood pressure accompanied by bradycardia, followed by a short lasting increase and than a long-lasting decrease of blood pressure after intravenous injection of veratridin or other veratrum alkaloids.

Kalkman et al. (1984) showed that three distinct subtypes of serotonergic receptors mediate the triphasic blood pressure response to serotonin observed in the Bezold-Jarisch reflex.

The Bezold-Jarisch reflex has been studied in several species, such as cats (Takei et al. 1995; Vayssettes-Couchay et al. 1997), dogs (Zucker and Cornish 1981; Barron and Bishop 1982; Harron and Kobinger 1984; Giles and Sander 1986; Baugh et al. 1989; Watson et al. 1995), ferrets (Andrews and Bhandari 1993), rabbits (Chen 1979), guinea pigs, rats (Fozard 1984; Gylys et al. 1988; Cohen et al. 1989; Blower 1990; Miyata et al. 1991; Turconi et al. 1991; Matsumoto et al. 1992; Meller et al. 1992; Robertson et al. 1992; Kishibayashi et al. 1993; Geissler et al. 1993; Haga et al. 1994; Hegde et al. 1994; Eglen et al. 1995; Göthert et al. 1995; Delagrange et al. 1996; De Vries 1997) and mice (Eglen et al. 1994; Middlefell et al. 1996), whereby species differences have been observed (Yamono et al. 1995).

In cats and dogs, the Bezold-Jarisch reflex was elicited by veratrine and veratridine, but also by capsaicin and the 5-HT_3 receptor agonists 2-methyl-5-HT, phenylbiguanide, chloro-phenylbiguanide and serotonin itself.

In rats, mostly 5-HT or 2-methyl-5-HT were used as stimuli to characterize 5-HT_3 receptor antagonists.

PROCEDURE

Male Sprague Dawley rats weighing 250–380 g are given food and water ad libitum, except those used for intraduodenal drug administration; these rats are de-

prived of food overnight. The animals are anesthetized by intraperitoneal injection of 1.5 g/kg urethane. Body temperature is maintained at 37 °C by placing the animal on a heating pad. The left jugular vein or duodenum, trachea and left femoral vein are cannulated for drug administration (i.v. or i.d.), facilitation of respiration and injection of 2-methyl 5-HT, respectively. Heart rate is derived from a limb lead II ECG monitored via subdermal platinum electrodes and is recorded with amplifiers on a polygraph. A dose-response curve to 2-methyl 5-HT (5–100 µg/kg, i.v.) is constructed in each rat to establish a submaximal dose (usually 10 or 20 µg/kg, i.v.) which elicits a reproducible bradycardic response. Each rat receives then a single dose of test drug or standard and is then challenged with 2-methyl 5-HT at 5, 15, 30, 60, 120, 180, 240, 300, 360, 420, and 480 min post dosing. A separate group of rats receiving vehicle (saline for i.v., deionized water for i.d.) is similarly tested in each study.

EVALUATION

Duration of action of the compounds is assessed by determining the period of time for which the inhibitory effects remain significantly different from vehicle controls. Statistical analysis of the data is performed by a repeated measure analysis of variance (ANOVA) followed by pairwise comparisons against control at each time period using Fisher's LSD multiple comparison test.

MODIFICATIONS OF THE METHOD

Harron and Kobinger (1984) used capsaicin to elicit the Bezold-Jarisch reflex in anesthetized artificially respirated dogs pretreated with a beta-adrenoceptor antagonist to evaluate the activity of clonidine-like drugs on central alpha$_2$ adrenoceptors after intracisternal administration.

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A.1.3.20 Endotoxin induced shock

PURPOSE AND RATIONALE

Many bacterial infections as well as allergic reactions are known to induce pathophysiological events that may lead to shock in man. When experimental animals are injected with endotoxin and galactosamine, shock and death occur in all untreated animals 5–7 h after injection. The endotoxin induced shock is marked by pulmonary embolism, bronchospasm and renal failure. Bacterial liposaccharides (endotoxins) play an important role in the pathogenicity of Gram-negative infections.

The reactivity of animals to endotoxin may be enhanced by simultaneous administration of galactosamine. Galactosamine is a specific hepatotoxic agent that leads to early metabolic alterations and consequent cellular liver damage. The following procedure is used to detect compounds that prevent the occurrence of endotoxin-induced shocks.

Cardiovascular parameters of endotoxin induced shock are greatly influenced by various anesthetics. For this reason, a model was proposed by Brackett et al. (1985) and Schaefer et al. (1987) to study the circulatory shock pattern after endotoxemia in conscious unrestrained rats.

PROCEDURE

Male Sprague-Dawley rats weighing 300 ±10 g are anesthetized with 5% enflurane. A tracheal cannula is connected to a rodent respirator delivering 2% enflurane. Via the right jugular vein the tip of one catheter is placed just adjacent to the right atrium for injection of endotoxin, monitoring of central venous pressure, and rapid injection of room-temperature saline to produce thermodilution curves for calculation of cardiac output. The right carotid artery is cannulated with a thermistor-catheter combination for measurement of thermodilution cardiac output curves and aortic blood pressure. The thermistor tip is placed in the aortic arch just distal to the aortic valve. The catheters are guided under the skin exiting through the back of the neck just below the base of the skull.

The animals are allowed to regain consciousness and are then placed in cages that allow unrestrained movements about the cage at all times throughout the study with no further handling. The experimental animals receive a 20-sec infusion of 40 mg/kg endotoxin (*E. coli*, Difco) being paired with sham animals with identical catheters but receiving an equal volume of saline. Test compounds are injected intravenously 10 min prior to endotoxin injection. Cardiac outputs are measured using the thermodilution technique by rapidly injecting a volume calculated to deliver 100 µl of room temperature saline to the circulatory system. Central venous and aortic blood pressure and heart rate are continuously monitored for the following 4 h. Cardiac output measurements are made 5, 15, 30, 60, 120, 180, and 240 min after endotoxin. At the end of the study, the animals are sacrificed and the catheters checked visually to ensure proper placement.

EVALUATION

Central venous pressure, arterial pressure, and cardiac output of drug treated animals receiving endotoxin are compared with animals receiving endotoxin only and saline sham treated animals. Furthermore, cardiac index, total peripheral resistance, and stroke volume are calculated. The small intestines of all rats are examined for severity of hemorrhage using a five point scale. Repeated-measures analysis of variance is used to analyze the data.

MODIFICATIONS OF THE METHOD

Lindenbaum et al. (1990) studied the effect of *E. coli* endotoxin on cardiovascular parameters of anesthetized dogs. Inhibition of the detoriation of metabolic functions and improvement of cardiovascular parameters were found after cocarboxylase treatment.

Endotoxin induced shock has been tested in **mice** (Galanos et al. 1979). Groups of 10 male C57 BL/6 mice weighing 20–22 g are injected intravenously with a mixture of 0.01 µg of Salmonella abortus equi lipopolysaccharide and 7.5–15 mg galactosamine in 0.02 ml phosphate buffered saline. The test compound is administered either intravenously at the same time or orally 45 min prior challenge. Twenty-four hours later, the number of surviving mice is determined.

Metz and Sheagren (1990) reviewed the effects of ibuprofen in animal models of septic shock.

Baldwin et al. (1991) tested the effect of Polymyxin B on experimental shock from meningococcal lipooligosaccharide and *Escherichia coli* lipopolysaccharide endotoxins in anesthetized **rabbits**.

Muacevic and Heuer (1992) tested the effect of platelet-activating factor antagonists in anesthetized rats.

Otterbein et al. (1993) tested the effects of peptides on survival of mice injected with 50 mg/kg lipopolysaccharide endotoxin in mice and on survival of rats with fecal peritonitis.

Mountz et al. (1995) reported an increased susceptibility of fas mutant **MRL-Ipr/Ipr mice** to staphylococcal enterotoxin B-induced septic shock.

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A.1.3.21 Hemorrhagic shock

PURPOSE AND RATIONALE

Hemorrhagic shock is one of the most severe consequences of accidents. Several animal models in various species have been developed to resemble the conditions in man and to test therapeutic or prophylactic measures (Lamson and de Turk 1945; Selkurt and Rothe 1961; Mills 1976). A method for hemorrhagic shock in anesthetized as well as in unanesthetized rats has been described by van der Meer et al. (1987). Experimental hemorrhagic shock is defined as a situation in which the cardiovascular system, after a period of hypovolemia followed by complete re-infusion of the shed blood, gradually detoriates ending in the death of the animal.

PROCEDURE

Female rats weighing 170–190 g are anesthetized by i.p. injection of sodium pentobarbital, 25 m/kg, followed after 20 min by 20 mg/kg, and kept in a chamber at 30 °C and relative humidity over 80%. The left femoral vein is cannulated for application of the test drug. The right common iliac artery is cannulated and the cannula (polyvinyl chloride, 14 cm long, inner diameter 2 mm) is filled with heparin and exteriorized in the neck. After intraarterial injection of 0.2 ml heparin 500 IU/ml, the cannula is connected to a siliconized calibrated glass reservoir (inner diameter 18 mm), the height of which can be changed to adjust the surface of the shed blood to a fixed level.

The test drug is injected i.v. 5 min prior to bleeding. Bleeding is performed against (at heart level) 30 mm Hg for 1 h, 25 mm Hg for 0.5 h, 30 mm Hg for 1 h, 25 mm Hg for 0.5 h, and finally 30 mm Hg for 1 h. The shed blood is partially taken up again spontaneously. After 4 h, re-infusion is started by increasing the pressure to 60 mm Hg for 5 min, to 80 mm Hg for 5 min, and (if necessary) to 100 mm Hg. During the hypovolemic phase respiration becomes gradually slower. If respiration arrest is imminent 0.5 ml 5% glucose are injected intra-arterially, thus avoiding death during the period of hypovolemia. Practically all rats die at an average of 4 h after complete re-infusion.

EVALUATION

Survival time is taken as the time between complete reinfusion and death. Average survival time of treated animals is compared with that of controls. Furthermore, after autopsy the number of gastrointestinal lesions, subendocardial hemorrhage, kidney tubular necrosis and liver cell necrosis are registered by histological examination.

CRITICAL ASSESSMENT OF THE METHOD

In spite of the fact that hemorrhagic shock does not reflect the situation of traumatic shock in man in every aspect, the condition is close enough to use the model for testing compounds which potentially inhibit or ameliorate shock in man.

MODIFICATIONS OF THE METHOD

A method to study hemorrhagic shock in dogs has been described in detail by Mills (1967). Large dogs weighing 20–30 kg are anesthetized by an i.v. injection of 25 mg/kg sodium pentobarbital. The animals are respirated by means of a Harvard respirator set at a stroke volume of 400 ml and a rate of 20 respiration/min. Blood pH is regulated between 7.37 and 7.42 by varying the gas flow between 100% O_2 and a mixture of 95% O_2 and 5% CO_2. Central arterial blood pressure is recorded by inserting a catheter through one femoral artery to the aortic arch. Pulmonary artery pressure is measured by inserting a PE 50 catheter through a small neck vein, reaching the right ventricle and allowing to float into the pulmonary artery. The right atrial catheter is also inserted through a small neck vein. After the chest is opened, the left atrial catheter is tied in place through a small opening in the left atrial appendage. Blood flow is measured in the ascending aorta (cardiac output), carotid, superior mesenteric, renal and femoral arteries using electromagnetic flowmeters. Furthermore, pulse rate is monitored from the electrocardiogram.

The test drug is injected i.v. 10 min prior bleeding. Blood is removed either at a specific volume or until a selected reduction of blood pressure has occurred. The cardiovascular parameters of treated animals are compared with those of controls.

Shock associated with hemoconcentration was produced in dogs by Davis (1941) by bleeding from the carotid artery and injections of 25% sodium chloride solution subcutaneously in doses of 25 ml.

The effect of insulin on glucose uptake in the soleus muscle of rats during hemorrhagic shock was studied by Chaudry et al. (1975).

Bauer et al. (1995) used hemorrhagic shock in rats to evaluate the influence of interleukin-1 on leukocyte-endothelial cell interactions and the microcirculation in the liver by means of intravital microscopy after application of an interleukin-1 receptor antagonist.

Kitajima et al. (1995) studied gastric mucosal injury induced by hemorrhagic shock in rats.

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A.1.3.22 Tourniquet shock

PURPOSE AND RATIONALE

Compression of extremities in man by heavy objects for periods of several hours results in the so-called crush syndrome. The rescued individual shows immediately a favorable response to therapy, but within a few hours symptoms of shock develop followed by signs of progressive renal damage leading to death (Duncan and Blalock 1942). Moreover, arterial bleeding after accidents needs the applications of tourniquets. During surgical procedures on extremities a tourniquet may be necessary (Wilgis 1971), the time of which has to be limited in order to avoid fatal consequences. The pathophysiological mechanisms of tourniquet induced shock remain still to be elucidated. Nevertheless, animal models in rats (Chandra and Dave 1970), rabbits (Little 1974), and dogs (Goto et al. 1988) had to be developed to evaluate drugs capable to inhibit the fatal consequences of crush and tourniquet shock.

PROCEDURE

Wistar rats of either sex weighing 250–280 g are anesthetized with phenobarbital. The tourniquets consist of

rubber tubes (internal diameter 4 mm, external diameter 5.8 mm). Both tights are fastened by the rubber tubes and the pressure which is monitored by a miniature pressure sensor and an amplifier (e.g. Kyowa Electronic Instruments Co, Tokyo) is adjusted to 1.5 kg/cm^2. The rubber tubes are knotted and the sensor removed. After 3 h the animals are treated with the test compound or the control solution. The tourniquet is left in place for 6 h while the animals remain under pentobarbital anesthesia. Then, the rubber tubes are removed, and the rats are returned to their cages. Within a few min, the reperfused hind limbs, which have been pale blue, turn pink. The animals are then allowed free access to food and water. Blood is withdrawn at different intervals during the tourniquet and afterwards for measurement of hematocrit, transaminases, urea nitrogen and total protein. Time to death is registered.

EVALUATION

Statistical evaluation of the survival intervals is performed with the log rank test according to Peto et al. (1976). Blood chemical data are analyzed using the Kruskal-Wallis (1952) rank sum test. Multiple comparisons are corrected by the Bonferroni's method (1980).

CRITICAL EVALUATION OF THE METHOD

These methods are valuable to find drugs effective in this life-threatening situation.

MODIFICATIONS OF THE METHOD

Ghussen et al. (1979) studied the effect of methylprednisolone on the experimental tourniquet shock in **dogs**.

Haugan and Kirkebo (1984) used a model in anesthetized **rats** with tourniquet shock by bilateral hindlimb occlusion for 3 1/2 h, and burn shock by scalding the hind 50% of the body surface for 30 s in 90 °C water.

Horl and Horl (1985) investigated the effect of tourniquet ischemia on carbohydrate metabolism in **dog** skeletal muscle

Sáez et al. (1982) followed the time course of appearance of lactic dehydrogenase enzymes in the serum of **rats** after different periods of ischemia by bilateral application of rubber band tourniquets to the hind legs.

Sáez et al. (1986) studied the effects of allopurinol on biochemical changes of the gastrocnemius muscle in rats subjected to tourniquet shock followed by reperfusion.

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A.1.3.23 Heat stroke

PURPOSE AND RATIONALE

Heat stroke is a medical emergency where quick diagnosis and treatment of victims are essential for positive prognosis. Several animal models have been established by investigators in heat related studies. Rats (Francesconi and Mager 1978; Hubbard et al. 1977, 1979; Kielblock et al. 1982), rabbits (Shih et al. 1984), dogs (Bynum et al. 1977) and sheep (Tayeb and Mar-

zouki 1990) are considered to be the most suitable models because of their similarity to man in response to high temperature.

PROCEDURE

Male Sprague Dawley rats weighing 450 to 550 g are fasted 18–24 h before the experiment. For prevention studies the animals are treated subcutaneously 1 h before either being restrained in an appropriate wire cage which is placed into an environmental chamber set at 41.5 °C ambient temperature or being exercised in a motor-driven treadmill. Core temperature (rectal probe inserted 6.5 cm) are measured using copper/constantan thermocouples in conjunction with a thermocouple reference oven and a 10-channel data acquisition system with a teletype printout. After reaching exhaustion or a predetermined core temperature, all rats are monitored at 26 °C ambient temperature while resting in plastic cages lined with wood shavings. After recovery, animals are returned to their cages and allowed water but no food for 24 h.

EVALUATION

LD_{50} values are determined in treated and control animals.

MODIFICATIONS OF THE METHOD

Kielblock et al. (1982) analyzed cardiovascular function by direct recording of arterial blood pressure and ECG-analysis.

Francesconi and Mager (1978) studied pathochemical indices, such as serum lactate concentration, potassium levels and plasma creatine phosphokinase.

Kregel et al. (1988) investigated peripheral vascular responses to hyperthermia in the rat by implantation of Doppler flow probes on the superior mesenteric, left iliac or left renal, and external caudal arteries. They concluded that a selective loss of compensatory vasoconstriction triggers the cascade of events that characterize heat stroke.

Shido and Nagasaka (1990) studied thermoregulatory responses to acute body heating in rats acclimated to continuous heat exposure. Indirect external warming was performed by raising the jacket water temperature surrounding the calorimeter from 24 to 39 °C. Intraperitoneal heating was made through an electric heater implanted chronically in the peritoneal cavity.

Chiu et al. (1995) reported an increased survival in rat heatstroke by reducing hypothalamic serotonin release after administration of interleukin-1 receptor antagonist.

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A.1.3.24 α- and β-adrenoreceptors in the mouse iris

PURPOSE AND RATIONALE

A simple method to test mydriatic substances is the test on the mouse pupil as described by Pulewka (1932). The diameter of the pupil is narrowed by intensive light illumination. A dose-dependent increase of pupil diameter can be achieved by intraperitoneal application of atropine and synthetic mydriatics (Ing et al. 1950; Burn et al. 1950). The mydriatic effect of hexamethonium analogues has been measured by Blackman et al. (1956). Mydriasis is induced by norepinephrine, epinephrine and isoproterenol and can be antagonized by α- or β-blockers (Freundt 1965).

PROCEDURE

Male mice weighing 15–20 g are used. They are kept for at least 30 min in separate beakers under bright illumination before the pupil diameters are measured with a dissecting microscope containing an arbitrary scale in the eyepiece. To make the illumination as uniform as possible, the beakers containing the mice are placed beneath long low-power fluorescent tubes and on top of glossy white paper. The pupil diameter is measured in mm before and at various time intervals

after treatment. Groups of 5–10 mice are used for each dose of compound and for vehicle control.

To test sympatholytic activity, various doses of the α- or β-blocker are injected subcutaneously 30 min prior to intravenous injection of 0.1 mg/kg norepinephrine, or 0.05 mg/kg epinephrine, or 20 mg/kg isoproterenol. The effect of norepinephrine is blocked by α-blockers, but not by β-blockers, the effect of epinephrine by both α- and β-blockers, and the effect of isoproterenol by β-blockers, but not by α-blockers.

EVALUATION

The mean values of diameters in the groups treated with α- or β-blockers are compared with those of animals treated with norepinephrine, epinephrine or isoproterenol only.

MODIFICATIONS OF THE METHOD

Edge (1953) used mydriasis in the mouse as a quantitative method of estimating parasympathetic ganglion block.

Håkanson et al. (1987) used the isolated iris sphincter of pigmented rabbits to test multiple tachykinin pools in sensory nerve fibres. The eyes were taken out within 1 min after sacrifice and opened by an incision 2–3 mm posterior to the limbus, followed by excision of the iris from the ciliary margin. The iris sphincter muscle was then opened, cut in half and mounted vertically on a Perspex holder in a 7 ml tissue bath maintained at 35ºC. The mechanical activity after electrical stimulation was recorded isometrically using a force displacement transducer and a polygraph.

Kern (1970) used isolated sphincter and dilator muscles from human eyes obtained at autopsy for studies on sympathomimetics and adrenergic blocking agents. Cholinotropic and α- and β-adrenergic receptors were identified.

Responses to bradykinin and or capsaicin of the isolated iris sphincter were considered to be mediated by substance P released from the trigeminal nerve (Ueda et al. 1984).

Pupillary dilatation can be used as an index for central nervous system α_2-adrenoreceptor activation (Koss 1986).

Clonidine induces mydriasis which is mediated by α_2-adrenoreceptors located in the brain (Berridge et al. 1983; Hey et al. 1985). Blockade of presynaptically located α_2-adrenoreceptors is considered as a possible mechanism for antidepressant drugs. Mianserin was able to antagonize clonidine-induced mydriasis in the rat.

Gower at al (1988) studied a large number of psychotropic drugs in this model with the aim to reveal *in vivo* α_2-adrenoreceptor blocking effects of new compounds.

Male Wistar rats weighing 230–300 g were anesthetized with pentobarbital, 60 mg/kg i.p., and a polyethylene catheter was inserted into the femoral vein for drug administration. The rat's head rested on the base platform of a binocular Olympus microscope positioned so that the pupil diameter of the right eye could be measured by means of a micrometer inserted into one eyepiece of the microscope. A constant light intensity was maintained throughout the experiment. Rats were first injected with saline 25 min after anesthesia induction. The pupil diameter was measured 1 min after injection. Five min after measurement, mydriasis was induced by clonidine (0.1 mg/kg, i.v.) and the diameter was measured 1 min after injection. This was followed by the test compound, injected at 6 min intervals at increasing doses. The pupil was measured at 1 min after each injection. The dose inhibiting 50% of the clonidine-induced mydriasis (ID_{50}) was determined per rat from the cumulative dose-response curve.

Savontaus et al. (1997) studied the effect of an imidazoline derivative against detomidine-induced mydriasis in anesthetized rats.

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A.1.3.25
α_2-adrenoreceptor blockade measured *in vivo* by clonidine-induced sleep in chicks

PURPOSE AND RATIONALE
In young chicks, clonidine causes a loss of righting reflex which is antagonized by mianserin (Pommier et al. 1982). This phenomenon was used to measure α_2-adrenoceptor blockade *in vivo* by Gower et al. (1988).

PROCEDURE
Male white Leghorn chicks are used either a few hours after hatching or 1 or 2 days later. Clonidine-induced loss of righting reflex (sleep) is determined with 8 animals at a time. Two animals are treated with placebo and 2 with each of 3 dose levels of the test compound. Tests with groups of 8 animals are continued until 10 animals are tested per dose level or placebo treatment. The chicks are marked with ink and injected intraperitoneally with placebo or the test compound. Ten min later, 1.2 mg/kg clonidine is injected into a leg muscle and the animals are placed individually in small Macrolon cages. The beginning of sleep time is defined as the moment at which the animals can be placed on their back and remain in this position. Sleep time is recorded until they return to their feet spontaneously or another attempt to put them on their back fails. Sleep time is recorded for a maximum period of 30 min.

EVALUATION
Statistical evaluations of differences in median sleeping times are done with the Mann-Whitney *U*-test. Dose-response relations for various drugs can be calculated.

CRITICAL ASSESSMENT OF THE METHOD
Compounds with known α_2-adrenoceptor blocking activity antagonize clonidine-induced sleep in chicks dose-dependently. Yohimbine is one of the most active compounds. However, also other centrally active compounds of which their main effect is not α_2-blockade, reduce clonidine-induced sleeping time. One of the most potent is apomorphine acting on dopamine D_2-receptors. Therefore, the clonidine-induced sleeping test in chicks can not be regarded as highly specific for α_2-adrenoceptors.

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A.1.3.26
Activity at β_1- and β_2-adrenoreceptors in the rat

PURPOSE AND RATIONALE
The relative potency of catecholamines as stimulants of β-adrenoreceptor mediated responses vary in different tissues indicating the existence of two subtypes of β-receptors (β_1 and β_2) (Lands et al. 1967). β-adrenoreceptors in the heart have been classified as being of the β_1-subtype. β-adrenoreceptors in the uterus, diaphragm, bronchioles and small intestine have been classified as being of the β_2-subtype, since in these tissues, epinephrine is more potent than norepinephrine. These observations led to the development of selective agonists and antagonists. Isolated organs (see below) having predominantly one receptor subtype, such as the isolated heart and the isolated atrium for β_1, and the isolated uterus or the isolated tracheal chain for β_2, are used to test compounds for selective activity. Assessing both activities in the same animal *in vivo* results in the advantage that pharmacokinetic and metabolic influences of the drug being tested are the same for both parameters.

PROCEDURE
Female Sprague-Dawley rats (200–220 g) are anesthetized with 60 mg/kg pentobarbital i.p. prior to pithing (Gillespie and Muir 1967). The animals are artificially respired with room air using a Harvard small animal ventilator (90 strokes/min at a pressure of 7 cm H_2O). Body temperature is maintained by placing the animals on a heated operating table. The left carotid artery is cannulated for continuous monitoring of blood pressure via a Statham p23Id pressure transducer. The

blood pressure signal is used to trigger an instantaneous rate meter for continuous monitoring of heart rate. A femoral vein is cannulated for intravenous administration of drugs.

A midline incision is made to expose one horn of the uterus. The ovarian artery is cut, tied and one horn dissected free from the ovary leaving the myometrial blood supply intact. A cotton thread is attached to the free end of the uterine horn, passed through a glass-jacketed organ bath and connected to an isometric (Pioden UF1) transducer for measurement of spontaneous contractions. A cannula is inserted into the peritoneal cavity for administration of drugs by the i.p. route. The organ bath is positioned such that it surrounds the uterine horn without touching it. The tissue is perifused with Krebs-Henseleit solution being gassed with 95% O_2/5% CO_2 and maintained at 37 °C. A resting tension of 0.2 g is applied to the tissue, which is allowed to stabilize until spontaneous contractions are constant over a period of 5–10 min. All recordings are made on a polygraph.

EVALUATION OF AGONISTS

Dose-response curves after i.v. injection are established for isoprenaline (nonselective between β_1- and β_2-adrenoreceptors), salbutamol (selective for β_2-adrenoreceptors), and noradrenaline (selective for β_1-adrenoreceptors) in increasing heart rate (beats/min) and decreasing the height of uterine contraction (calculated as percentage of the original amplitude). Animals given noradrenaline are pretreated with phenoxybenzamine (3.3 mmol/kg i.v.) in order to antagonize irreversibly the α-adrenoreceptors. Agonist dose-response curves ($n > 4$) on heart rate and uterine relaxation are carried out by assessing the activity of at least 3 doses of each agonist. New synthetic compounds can be tested after intraperitoneal administration additionally.

EVALUATION OF ANTAGONISTS

The ability of a non-selective β-blocker, such as propranolol (1 mmol/kg i.v.), a β_1-selective β-blockerββ, such as atenolol, and a β_2-selective β-blockerββ to inhibit responses to isoprenaline on both heart rate and uterine relaxation is assessed by comparing the log linear portion of the dose-response curve to isoprenaline in the absence and in the presence of the β-adrenoreceptor antagonist in the same animal. Dose ratios for each antagonist are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method described by Piercy (1988) has the advantage to measure both agonistic and antagonistic activity and to differentiate between effects on β_1- and β_2-adrenoreceptors. Compared to tests in isolated organs, *in vivo* activity can be determined after intraperitoneal or intraduodenal administration.

MODIFICATIONS OF THE METHOD

Härtfelder et al. (1958) studied the influence of various agents on the contractions of electrically stimulated **isolated uteri of rabbits and guinea pigs**.

Nathason (1985) evaluated the activity of beta-blockers to inhibit the cardio-acceleratory effect of systemically administered isoproterenol in **unanesthetized, restrained albino rabbits** together with the effect on membrane bound adenylate cyclase in homogenized ciliary process villi in order to find compounds selectively lowering intraocular pressure.

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A.1.3.27
β_1- and β_2-sympatholytic activity in dogs

PURPOSE AND RATIONALE

Intravenous administration of isoprenaline (isoproterenol) stimulates β_1-receptors of the heart which can be detected as an increase in contractility (*dp*/*dt* max). Intraarterial injection of isoprenaline stimulates β_2-receptors of peripheral blood vessels leading to an increased peripheral blood flow. Therefore, a β_1- or β_2-blocking activity of a compound is revealed by the inhibition of the effects of isoprenaline. The following tests are used to evaluate β-blocking activity of drugs. A β-blocker screening is done in anesthetized dogs (a); in addition, the test allows a differentiation between

β_1- and β_2-receptor activity and the determination of ED_{50} values (b).

PROCEDURE

Male or female Beagle dogs weighing about 20 kg are used. Animals are premedicated with 1 g Inactin® (i.v.) and anesthetized by intravenous administration of 20 mg/kg chloralose and 250 mg/kg urethane. In addition, they receive a subcutaneous injection of 2 mg/kg morphine 1 h after the start of anesthesia. Animals are heparinized. Respiration is maintained through a tracheal tube using a positive pressure respirator. End-expiratory CO_2 content is measured continuously; respiratory rate and depth of respiration are adjusted to 4.5–6 vol% end-expiratory CO_2. For administration of isoprenaline, a peripheral vein is cannulated.

Preparation for hemodynamic measurements

For recording of peripheral systolic and diastolic blood pressure, a cannula inserted into a femoral artery is connected to a pressure transducer (Statham p 23 DB). For determination of LVP, a Millar microtip catheter (PC 350) is inserted via the left arteria carotis communis. LVEDP is measured from a high-sensitivity scale. From the pressure curve, *dp*/*dt* max is differentiated and heart rate is counted. Peripheral blood flow in the femoral artery is measured with an electromagnetic flow probe.

Screening for β-blocking effects in anesthetized dogs

Following a steady-state period of 30–60 min, isoprenaline is administered intravenously 2–3 times to the anesthetized animal and hemodynamic parameters are recorded (control values = 100%). Then, the test substance is injected intravenously at cumulative doses (final concentrations of 0.01, 0.05 and 0.15 mg/kg). For each dose, 10 min "drug effects" are monitored by measuring hemodynamic parameters. Then the effect of isoprenaline is tested again (3 times).

In other experiments, a single dose of the drug is administered to determine the duration of action.

If a test compound does not show an inhibitory influence on isoprenaline effects, a second test compound is administered.

All hemodynamic parameters are registered continuously during the whole experiment.

Testing for β_1- and β_2-blocking effects; determination of ED_{50}

Following a steady-state period of 30–60 min, isoprenaline is administered for i.v. administration (β_1-test) twice at a dose of 0.5 µg/kg and for intraarterial administration (β_2-test) twice at a dose of 0.05 µg/kg. Hemodynamic parameters are recorded (control values = 100%). Then, the test substance is injected intravenously at cumulative doses. Consecutively increasing doses are given at 15 min-intervals. For each dose, 10 min "drug effects" are monitored by measuring hemodynamic parameters. Thereafter isoprenaline is given intravenously and 5 min later intra-arterially.

All hemodynamic parameters are registered continuously during the whole experiment.

Characteristics:
- blood pressure
  - systolic, BPs
  - diastolic, BPd
- heart rate
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure, LVEDP
- *dp*/*dt* max
- peripheral flow, A. femoralis
- ECG, lead II

Standard compounds:
- propranolol HCl
- practolol
- metoprolol tartrate

EVALUATION

β_1-receptor antagonism is measured as a decrease in contractility (*dp*/*dt* max).

Inhibition of the isoprenaline-induced elevation of heart rate is considered as an indicator for non-selective β-blockade. For cardioselective β-receptor blockers the increase in *dp*/*dt* max is inhibited with lower doses of test drug than the rise in heart rate.

β_2-receptor blockade by a test drug is measured as inhibition of the isoprenaline-induced increase in peripheral blood flow.

The different hemodynamic parameters are determined.

Percent inhibition of the isoprenaline-induced effects by a test compound is calculated and compared to the isoprenaline effects before drug administration (= 100%).

ED_{50} values for β_1- and β_2-antagonism are calculated by log-probit analyses. ED_{50} is defined as the dose of drug leading to a 50% inhibition of the isoprenaline effects.

An ED_{50} β_1/ED_{50} β_2-ratio of >1 indicates that a β-blocking agent predominantly influences β_1-receptors (cardioselectivity).

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A.1.3.28 Intrinsic β-sympathomimetic activity in reserpine-pretreated dogs

PURPOSE AND RATIONALE

β-blocking agents can be classified as

- β-blocking agents with intrinsic sympathomimetic activity (ISA),
- β-blocking agents with membrane stabilizing activity (MSA),
- β-blocking agents with organ selectivity (high affinity to heart β_1-receptors).

In the following procedure with reserpine-pretreated dogs, β-blocking agents with intrinsic sympathomimetic activity can be identified. Reserpine administration 24 h before the start of the experiment leads to a depletion of catecholamine depots. Thus, it is possible to differentiate between indirectly acting sympathomimetics such as tyramine and directly acting ones such as noradrenaline.

This test is used to identify β-blocking drugs with intrinsic sympathomimetic activity.

PROCEDURE

Male or female Beagle dogs weighing about 15 kg are used. Twenty-four h before the test, dogs receive an intramuscular injection of 0.3 mg/kg reserpine. At the day of the experiment, the animals are anesthetized by intravenous administration of 10–20 mg/kg pentobarbital sodium. Respiration through a tracheal tube using a positive pressure respirator is controlled by measuring end-expiratory CO_2 concentrations (4–5 vol%).

Preparation for hemodynamic measurements

For recording of peripheral systolic and diastolic blood pressure, a femoral artery is cannulated and connected to a pressure transducer (Statham p 23 DB). For determination of LVP, a Millar microtip catheter (PC 350) is inserted into the left ventricle via the left common carotid artery. LVEDP is measured from a high-sensitivity scale. From the pressure curve, *dp/dt* max is differentiated and heart rate is counted.

Experimental course

The test substance is administered by continuous intravenous infusion of 0.02 mg/kg (1 ml/min) until a cumulative dose of 3 mg/kg is achieved (within approximately 150 min). Thereafter, the velocity of infusion is doubled (0.04 mg/kg, 2 ml/min). The test is finished when a cumulative dose of 7 mg/kg is achieved (after a total time of approximately 250 min).

Hemodynamic parameters are registered continuously during the entire experiment.

Characteristics:

- blood pressure
  - systolic blood pressure
  - diastolic blood pressure
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure, LVED
- *dp/dt* max
- heart rate, HR

EVALUATION

The different hemodynamic parameters are determined. As a measure for intrinsic sympathomimetic activity (ISA), the increase in *dp/dt* max and in heart rate are evaluated. Absolute and relative differences of these parameters in drug-treated animals are compared to vehicle control values.

Statistical evaluations are performed by means of the Student's *t*-test if $n > 4$.

Scores are allotted relative to the efficacy of standard compounds for intensity as well as for duration of the effect.

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A.1.3.29 Cat nictitating membrane preparation (ganglion blocking activity)

PURPOSE AND RATIONALE

Nicotinic acetylcholine receptors are involved in the ganglionic neurotransmission. Various subtypes are described for nicotinic acetylcholine receptors (Sargent 1993; McGehee and Role 1995; Karlin and Akabas 1995; Alexander et al. 2001).

The nictitating membrane of the cat has been used extensively in pharmacological studies to evaluate ganglion blocking activity because of the ease with which its movements can be recorded, because of the simplicity of its innervation (the purely adrenergic fibres have their cell bodies in the easily accessible superior cervical ganglion of the same site) and because its blood supply (via the external carotid artery) is accessible for intraarterial injections. Preganglionic and

postganglionic stimulation allow the interpretation of the mode of action of vasoactive drugs.

PROCEDURE

The animal is anesthetized with 35 mg/kg pentobarbital sodium i.p. Tracheostomy is performed and a tracheal cannula is inserted. On one side, the sympathetic nerve is exposed, separated from the vagus nerve and prepared in order to place electrodes for preganglionic and postganglionic stimulation. Preferably, the vagus nerve at this site is severed at the central end. The head of the animal is fixed in a head holder to prevent head movements. A linear transducer is fixed at the mid of the border of the nictitating membrane allowing the registration of the contractions on a polygraph. Preganglionic and postganglionic stimuli are exerted by a square wave stimulator, with a pulse width of 0.3 to 0.5 ms, an amplitude of 1–3 V, and a frequency of 20/min. The amplitude and pulse width varies from animal to animal. The sympathetic nerve is stimulated before and after the administration of the compound and the changes in the contraction of the nictitating membrane are noted. Furthermore, the response of the nictitating membrane to exogenous adrenaline is registered.

EVALUATION

The decrease of the response after drug application is expressed as percentage of the control before drug. Ganglionic blockers decrease the response to preganglionic stimulation but have no influence on postganglionic stimulation or exogenous adrenaline. Neuronal blockers decrease the response to both preganglionic and postganglionic stimulation but do not affect the response to exogenous adrenaline which may even be enhanced. α-receptor blockers decrease the response to both preganglionic and postganglionic stimulation as well as decrease the effect of exogenous adrenaline. Catecholamine uptake inhibitors increase the response to both preganglionic and postganglionic stimulation as well as enhance the response to exogenous adrenaline.

CRITICAL ASSESSMENT OF THE METHOD

The nictitating membrane preparation has been widely used for differentiation of cardiovascular effects. Since the use of higher animals such as cats has been limited to a great extent, this model is now being used only exceptionally.

As alternative, the contraction of the inferior eyelid of anesthetized *rats* after preganglionic electrical stimulation of the superior cervical ganglion has been recommended (Gertner 1956; Steinbrecher and Schmid-Wand 1986). In the modification used by Steinbrecher and Schmid-Wand (1986) the method is suitable for testing compounds with potential adrenergic and antiadrenergic activity but not for testing ganglion blocking activities.

Male Sprague Dawley rats are anesthetized with 100 mg/kg thiobutabarbital i.p. and kept on a heated operation table at a rectal temperature of 37 °C. One femoral vein is cannulated and filled with 4% heparin solution. One femoral artery is cannulated for registration of blood pressure. Tracheotomy is performed and a polyethylene catheter of 5 cm length inserted. The head of the animal is fixed carefully. The vibrissae at the lower eyelid on the right side are cut, a thread attached at the margin of this eyelid and attached to a strain-gauge. To immobilize the musculature of the face, the mouth of the animal is sutured and the head support attached. The right sympathetic nerve is exposed, separated from the vagus nerve and prepared in order to place electrodes for preganglionic stimulation. For calibration, stimulation is performed twice with an interval until contraction is back to baseline. Furthermore, a dose of 0.001 mg/kg adrenaline is given as bolus injection. Eyelid contraction and blood pressure increase are recorded. Then the putative adrenergic blocker or the standard 1.0 mg/mg phentolamine are injected intravenously. Eyelid contraction after electrical stimulation or after adrenaline is reduced dose-dependently.

CRITICAL ASSESSMENT OF THE METHOD

The nictitating membrane preparation has been widely used for differentiation of cardiovascular effects. Since the use of higher animals such as cats has been limited to a great extent, this model is now being used only exceptionally.

As alternative, the contraction of the inferior eyelid of anesthetized **rats** after preganglionic electrical stimulation of the superior cervical ganglion has been recommended (Gertner 1956; Steinbrecher and Schmid-Wand 1986). In the modification used by Steinbrecher and Schmid-Wand (1986) the method is suitable for testing compounds with potential adrenergic and antiadrenergic activity but not for testing ganglion blocking activities.

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to place electrodes for preganglionic stimulation. For calibration, stimulation is performed twice with an interval until contraction is back to baseline. Furthermore, a dose of 0.001 mg/kg adrenaline is given as bolus injection. Eyelid contraction and blood pressure increase are recorded. Then the putative adrenergic blocker or the standard 1.0 mg/mg phentolamine are injected intravenously. Eyelid contraction after electrical stimulation or after adrenaline is reduced dose-dependently.

MODIFICATIONS OF THE METHOD

Quilliam and Shand (1964) assessed the selectivity of drugs by comparing the effects on ganglionic transmission and on the pre- and post-ganglionic nerves in the isolated superior cervical ganglion preparation of the rat.

Langer and Trendelenburg (1969) performed experiments with normal nictitating membranes of pithed cats as well as with isolated normal nictitating membranes.

Koss and Hey (1992) used frequency-dependent nictitating membrane responses by sympathetic nerve stimulation in anesthetized cats to determine the potential role of prejunctional histamine H_3 receptors.

Gurtu et al. (1992) used contractions of the cat nictitating membrane to explore the effects of calcium channel blockers on neurotransmission *in vivo*, by comparing the effects of verapamil and nifedipine on contractions of nictitating membrane following either electrical stimulation of the superior cervical ganglion or intravenous injection of phenylephrine.

Koss (1992) compared the peripheral and central nervous system sympatholytic actions of prazosin using the cat nictitating membrane. Submaximal contractions of the nictitating membranes were evoked by electrical stimulation of the preganglionic cervical sympathetic nerve trunk and by stimulation of the posterior hypothalamus in anesthetized cats.

Badio et al. (1996) evaluated spiropyrrolizidines, a new structural class of blockers of nicotinic receptor channels with selectivity for ganglionic type receptors in rat pheochromocytoma PC12 cells (with an $\alpha_3\beta_{4(5)}$-nicotinic receptor) and human medulloblastoma TE671 cells (with an $\alpha_1\beta_1\gamma\delta$-nicotinic receptor).

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A.1.3.30
Assessment of ganglion-blocking activity in the isolated bovine retractor penis muscle

PURPOSE AND RATIONALE

The use of the bovine retractor penis muscle for the assessment of ganglion-blocking activity of neuromuscular blocking drugs has been recommended by Ala-

ranta et al. (1990) and Klinge et al. (1993). Klinge and Sjöstrand (1974) performed not only extensive studies on the physiology and pharmacology of the retractor penis in the bull, but also discussed the various hypotheses on inhibitory and excitatory innervation of this muscle, which is present in many vertebrates such as horses, cats, dogs and rats, but not in men and rabbits. They also found that the effects on the isolated retractor penis muscle and on penile arteries are rather similar. The excitatory innervation was found to be predominantly α-adrenergic (Klinge et al. 1970; Klinge and Sjöstrand 1977) whereas other transmitters such as histamine and bradykinin were effective only in some species. Relaxation of the isolated retractor penis muscle could be elicited by nicotine and other nicotinic agonists (Klinge et al. 1988). In the studies on ganglion-blocking activity, strips of the retractor penis muscle are precontracted by 5-hydroxytryptamine. Relaxation induced by nicotine is antagonized by ganglion-blockers.

PROCEDURE

Retractor penis muscles are obtained from bulls of different breeds weighing 250–500 kg. Samples are dissected 10–30 cm distal to the points where the paired muscle bundles pass the anal orifice. Immediately after slaughter, the samples are freed from fat and other surrounding tissue and placed into Tyrode solution at 2–4 °C. Strips, 15–25 mm in length and 2–3 mm wide, are prepared and mounted in 20-ml organ baths containing Tyrode solution at 35 °C aerated with 95% O_2 and 5% CO_2. An equilibrium time of 2 to 4 h is allowed. During the equilibrium period washed are performed at about 60-min intervals. Changes in tension are recorded by means of Grass FT 03 force displacement transducers coupled to a polygraph.

A high-enough tone for studying the nicotine-induced relaxation, usually 8–15 g, is generated by adding 5-HP in a concentration between 0.1 and 6 μM to the organ bath. Washing is performed 2 min after application of nicotine; 60–80 min later the tone is again raised and the application of nicotine is repeated. The effect of a neuromuscular blocking drug is studied only if the relaxations caused by nicotine in two consecutive controls are equal in size.

EVALUATION

The blocking activity of a certain concentration of a drug is expressed as % reduction in the relaxation of the muscle strip, according to the following equation:

$$\frac{A - B}{A} \times 100$$

Where A is the size of the control relaxation in millimeters, and B is the size of the relaxation of the blocking drug. In order to construct regression lines, the activity of four or five dose levels from the assumed linear part of the concentration-effect curve is studied. The activity of each dose level is studied in at least 5 strips obtained from different animals. IC_{50} values are calculated from the regression lines. The parallelism of the regression lines is tested by covariance analysis.

CRITICAL ASSESSMENT OF THE METHOD

Molar potency ratios of known ganglion-blocking agents obtained with this method were compared with the results of other methods, such as inhibition of contraction of cat nictitating membrane evoked by preganglionic sympathetic stimulation (Bowman and Webb 1972; see 1.3.29), inhibition of nicotine-induced contraction of the isolated guinea pig ileum (Feldberg 1951), inhibition of contraction of guinea pig vas deferens evoked by preganglionic stimulation of the hypogastric nerve *in vitro* (Birmingham and Hussain 1980), depression of postganglionic action potentials evoked by preganglionic stimulation of the superior cervical ganglion of the rat *in vitro* (Quilliam and Shand 1964), induction of mydriasis in mouse by blocking the ciliary ganglion (Edge 1953). A fair but not a complete agreement between the results obtained with various methods was found.

MODIFICATIONS OF THE METHOD

Gillespie and Sheng (1990) studied the effects of pyrogallol and hydroquinone on the response to non-adrenergic, non-cholinergic nerve stimulation in the rat anococcygeus and the bovine retractor penis muscles.

Parkkisenniemi and Klinge (1996) used samples of retractor penis muscles and penile arteries from bulls for functional characterization of endothelin receptors.

La et al. (1997) studied the inhibition of nitrergic nerve-induced relaxations in rat anococcygeus and bovine retractor penis muscles by hydroxycobalamin.

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A.1.3.31 Angiotensin II antagonism

PURPOSE AND RATIONALE

Angiotensin II antagonists can be tested in rats after elimination of cardiovascular reflexes by vagotomy and ganglionic blockade. Several angiotensin II antagonists possess intrinsic agonistic activity. This can be tested by injection of various doses to the vagotomized, ganglion-blocked animal. The antagonistic activity of the angiotensin II antagonist can be evaluated by antagonism against graded doses of angiotensin II. The duration of activity can be tested during continuous infusion of angiotensin II.

PROCEDURE

Male Sprague-Dawley rats weighing about 300 g are used. They are anesthetized with 60 mg/kg pentobarbital sodium i.v. One carotid artery is cannulated and connected with a Statham transducer P 23 Db. Blood pressure is recorded on a polygraph. Both jugular veins are cannulated for application of test compounds and for infusion. Both vagal nerves are cut 3 mm dorsal of the larynx. For ganglionic blockade, 10 mg/kg pentolinium tartrate are injected intravenously. At least 5 animals are used for evaluation of one test drug.

Intrinsic agonistic activity

After the blood pressure has reached a constant value, doses of 1, 2, 4 and 16 µg/kg of the test compound are injected via the jugular vein. Blood pressure is recorded.

Antagonistic activity

In 10 min intervals doses of 0.5; 1.0; and 2.0 µg/kg angiotensin II are injected to establish dose-response curves. After 10 min, continuous infusion is started of the potential angiotensin II blocker in a dosage of 10 µg/kg/0.1 ml/min. Ten min after beginning of the infusion, again doses of 0.5; 1.0; and 2.0 µg/kg angiotensin II are injected.

Duration of activity

In this set-up, angiotensin II is administered as continuous infusion at a dosage of 1 µg/kg/0.02 ml/min. When blood pressure has reached an elevated steady state level, 0.1 mg/kg of the angiotensin II antagonist is administered.

Intensity and duration of the fall of blood pressure are recorded.

EVALUATION

Intrinsic agonistic activity

An increase of blood pressure indicates the intrinsic agonistic activity.

Antagonistic activity

Increases of blood pressure after graduated doses of angiotensin II during the infusion is expressed as percentage of the increase before infusion. The results are compared with known angiotensin II antagonists.

CRITICAL ASSESSMENT OF THE METHOD

In this test not only potency and duration of activity but also the intrinsic agonistic activity of an angiotensin II antagonist can be tested.

MODIFICATIONS OF THE METHOD

Various other pharmacological models have been used to test angiotensin II antagonists:

Blood pressure in conscious unrestrained rats with chronically implanted catheters with normal blood pressure, spontaneous hypertension and chronic renal hypertension (Vogel et al. 1976; Chiu et al. 1989; Brooks et al. 1992; Aiyar et al. 1995; Deprez et al. 1995; Gabel et al. 1995; Hilditch et al. 1995; Keiser et al. 1995; Nagura et al. 1995; Nozawa et al. 1995; Renzetti et al. 1995; Wong 1995; Junggren et al. 1996),

Blood pressure in conscious spontaneously hypertensive and in anesthetized ganglion-blocked rats (Olins et al. 1993),

Blood pressure in pithed and in conscious renovascular hypertensive rats (Criscone et al. 1993; Wienen et al. 1993; Deprez et al. 1995; Kivlighn et al. 1995a; Kushida et al. 1995),

Blood pressure in rats after intracerebroventricularly injected angiotensin II (Vogel et al. 1976; Batt et al. 1988),

Blood pressure in conscious angiotensin I-infused and renin-dependent **hypertensive dogs** (Brooks et al. 1992; Cazaubon et al. 1993; Aiyar et al. 1995; Deprez et al. 1995; Gabel et al. 1995; Keiser et al. 1995; Wong et al. 1995),

Blood pressure and heart rate in conscious sodium-depleted and sodium-repleted **cynomolgus monkeys** (Lacour et al. 1993; Cazaubon et al. 1993; Keiser et al. 1995),

Angiotensin II induced pressor responses in **marmosets** (Nagura et al. 1995),

Blood pressure and heart rate in conscious **rhesus monkeys** and anesthetized **chimpanzees** (Gabel et al. 1995; Kivlighn et al. 1995b; Kivlighn et al. 1995c),

Inhibition of angiotensin II-induced contraction in isolated **aorta** rings or strips from **rabbits** (Chui et al. 1989, 1990; Criscione et al. 1993; Cazaubon et al. 1993; Olins et al. 1993; Wienen et al. 1993; Aiyar et al. 1995; Caussade et al. 1995; Hilditch et al. 1995; Keiser et al. 1995; Kushida et al. 1995; Nagura et al. 1995; Renzetti et al. 1995; Wong et al. 1995), from **rats** (Nozawa et al. 1997), from **neonatal rats** (Keiser et al. 1993), from **guinea pigs** (Mizuno et al. 1995),

Inhibition of angiotensin II-induced contraction in isolated rat pulmonary artery (Chang et al. 1995),

Antagonism against angiotensin II in isolated strips of rabbit aorta, rabbit jugular vein, rabbit pulmonary artery, rat portal vein, rat stomach, rat urinary bladder, human urinary bladder, human colon, human ileum (Rhaleb et al. 1991),

Contractions of **guinea pig ileum** *in situ* (Khairallah and Page 1961),

Antagonism against angiotensin II in the **isolated rat uterus** (Wahhab et al. 1993),

Contractile force and prostaglandin E synthesis in electrically stimulated **rabbit isolated vas deferens** (Trachte et al. 1990),

Antagonism against angiotensin II-induced aldosterone release in **bovine adrenal glomerulosa cells**. (Criscione et al. 1993), and in rat dispersed adrenal capsular cells (Chang et al. 1995),

Antagonism against angiotensin II-induced inhibition of guanylate cyclase activity in the **rat pheochromocytoma cell line** PC12W (Brechler et al. 1993).

Brooks et al. (1995) compared the cardiovascular and renal effects of an angiotensin II receptor antagonist and captopril in **rats with chronic renal failure** induced by 5/6 nephrectomy. Under sodium pentobarbital anesthesia the right kidney was removed and approximately two thirds of the left kidney was infarcted by ligating two or three branches of the left renal artery.

Kim et al. (1997) studied the effects of an angiotensin AT_1 receptor antagonist on volume overload-induced cardiac gene expression in rats. An abdominal aorta-caval shunt was prepared in 9-weeks old male Wistar rats under sodium pentobarbital anesthesia. The vena cava and the abdominal aorta were exposed by opening the abdominal cavity via a midline incision. The aorta was punctured at the union of the segment two thirds caudal to the renal artery and one third cephalic to the aortic bifurcation with a 18-gauge disposable needle. The needle was advanced into the aorta, perforating its adjacent wall and penetrating the vena cava. After the aorta was clamped, the needle was withdrawn, and a drop of cyanoacrylate glue was used to seal the aortic puncture point. The patency of the shunt was verified visually by swelling of the vena cava and mixing of arterial and venous blood. The rats were treated either with vehicle or the angiotensin antagonist. Four days after the preparation of the AC shunt, 24 h-urine volume, electrolytes and aldosterone were measured. Six days after the AC shunt blood was collected by puncture of a tail vein and plasma renin activity and aldosterone were measured. Seven days after AC shunt, hemodynamic studies were performed in pentobarbital anesthesia. Afterwards, the heart was rapidly excised, left and right atria and ventricles were separated and frozen in liquid nitrogen for the extraction and measurement of cardiac tissue RNA.

Shibasaki et al. (1997) tested the effect on the renin-angiotensin-aldosterone system in **conscious rats** after cannulation of the abdominal aorta under anesthesia 3–4 days before the experiment. After oral dosing of the angiotensin II receptor antagonist blood samples were withdrawn and plasma renin and aldosterone determined by radioimmunoassay.

Similar to the effects of ACE inhibitors, lifespan of hypertensive rats could be doubled by long-term treatment with an angiotensin II type 1 receptor blocker (Linz et al. 2000).

Ledingham and Laverty (1996) treated **genetically hypertensive New Zealand rats** with a specific AT_1 receptor antagonist via osmotic mini-pumps for several weeks and measured the effects on blood pressure, cardiac hypertrophy and the structure of resistance arteries.

Transgenic animals were recommended for further studies to influence the human renin-angiotensin system (Müller et al. 1995; Wagner et al. 1995; Bohlender et al. 1996).

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A.1.3.32
ACE inhibition measured *in vivo* in the rat

PURPOSE AND RATIONALE

The angiotensin-converting enzyme (ACE) is responsible for the cleavage of the almost inactive angiotensin I to the active angiotensin II. The same enzyme (kininase II) is responsible for the degradation of the active peptide bradykinin to inactive products. ACE activity can therefore be measured in two ways: activity of the newly formed angiotensin II and diminution of the activity of bradykinin. ACE inhibition results in decreased activity of the precursor angiotensin I and potentiation of the bradykinin effect. The cardiovascular system is sensitive to both peptides, reacting with an increase of blood pressure to angiotensin II and with a decrease to bradykinin. These reactions can be used for quantitative determination of ACE inhibiting activity.

PROCEDURE

Male Sprague-Dawley rats weighing 300–400 g are used. The animals are anesthetized by i.p. injection of 70 mg/hg pentobarbital. After intubation of the trachea they are artificially respirated with 30 strokes/min and a stroke volume of 6–8 ml. The right carotid artery is cannulated and blood pressure registered with a Statham-element (P 23 Db) and a polygraph. One jugular vein is cannulated for i.v. injections. After laparotomy a catheter is inserted into the duodenum for enteral administration and the wound closed again. Blood pressure is stabilized 30% below the normal level by i.m. injection of 5 mg/kg pentolinium. In order to prevent excessive mucus production in the bronchial system, 40 µg/kg atropine sulfate are injected intramuscularly.

Inhibition of angiotensin I cleavage

After stabilization of blood pressure, 310 ng/kg angiotensin I is injected intravenously in 0.1 ml saline. The injection is repeated in 5-min intervals until an identical pressure reaction occurs. The test compounds are administered at doses of 1 and 10 mg/kg intravenously or 25 mg/kg intraduodenally. 3 min after iv. injection or 10 min after i.d. administration, again 310 ng/kg angiotensin I is injected. Standards are ramipril, enalapril or captopril.

Potentiation of bradykinin-induced vasodepression

A low dose of bradykinin has to be chosen in order to visualize the bradykinin potentiation. One µg/kg, eventually 3 µg/kg bradykinin are injected intravenously at 5 min intervals until a stable reaction is achieved. Three min after i.v. injection or 10 min after intraduodenal administration of the test substance, the bradykinin injection is repeated.

EVALUATION

Inhibition of angiotensin I cleavage

The diminution of the pressure reaction to angiotensin I after administration of a potential ACE inhibitor is the parameter for the activity of the new compound. The inhibition is calculated as percent of controls. Using various doses of the ACE inhibitor, dose-response curves can be established and ID_{50} values be calculated.

Potentiation of bradykinin-induced vasodepression

Potentiation of bradykinin induced vasodepression is expressed as percentage of controls. Using various doses of the test compound and the standard, dose-response curves can be established and potency ratios calculated.

CRITICAL ASSESSMENT OF THE METHOD

Both parameters, inhibition of angiotensin I response and potentiation of bradykinin-induced vasodepression have been proven as reliable parameters for evaluation of ACE inhibitors.

MODIFICATIONS OF THE METHOD

Natoff et al. (1981) used the ratio of responses to angiotensin I and angiotensin II in spontaneously hypertensive rats, either pithed or anesthetized with urethane, to determine the degree and the duration of effect of captopril.

Blood levels of angiotensin II can also be measured by radioimmunoassay.

Several studies in rats showed the beneficial effects of prolonged treatment with ACE inhibitors. Postoperative mortality in rats with left ventricular hypertrophy and myocardial infarction was decreased by ACE inhibition (Linz et al. 1996).

Inhibition of angiotensin I-induced pressure response by administration of ACE-inhibitors can be measured not only in anesthetized rats, but also in anesthetized dogs, conscious rats and conscious dogs (Becker et al. 1984).

Life-long ACE inhibition doubles lifespan of hypertensive rats not only if the treatment is started at the age of one month (Linz et al. 1997), but ramipril also increases survival in old spontaneously hypertensive rats if treatment is started at the age of 15 months (Linz et al. 1999).

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A.1.3.33 Evaluation of renin inhibitors in dogs

PURPOSE AND RATIONALE

Highly specific inhibitors of the enzyme renin are considered to be potential antihypertensive agents. These agents cause a fall in blood pressure of sodium-deficient dogs and decrease plasma renin activity as well as angiotensin II level.

PROCEDURE

Animal experiment

Adult mongrel dogs (8–14 kg) of either sex are given water ad libitum and maintained on a low sodium diet for 1–2 weeks before the experiment. A single intramuscular injection of 5 mg/kg furosemide is given 48 h before the experiment. On the day of the experiment the dogs are anesthetized with sodium pentobarbital (30 mg/kg i.v.) and a cuffed endotracheal tube is positioned to allow artificial respiration. To measure arterial blood pressure a femoral artery is catheterized with polyethylene tubing. The right and left femoral veins are catheterized for drug administration and delivery

of a maintenance infusion of sodium pentobarbital (5 mg/kg/h). Blood pressure is measured directly through the catheter which is connected to a Gould-Statham pressure transducer. Blood samples are collected from the arterial catheter.

Increasing doses of the potential renin inhibitor are infused over 30 min followed by a 30 min recovery period. Immediately after the last recovery period, the dogs are given an i.v. infusion of the angiotensin receptor antagonist saralasin (20 µg/kg/min) for 30 min. For measurement of plasma renin activity and angiotensin II levels, the dogs are infused over a period of 30 min with the test compound and blood is withdrawn at 0, 15, 30, 60, 90, 120, 180, and 240 min after the start of the infusion. After the final blood drawing, 20 µg/kg/min saralasin is infused for 30 min.

Analytical procedures

The antibody-trapping method is preferred to measure plasma renin activity (PRA). In this procedure PRA is determined at pH 7.4 by RIA quantification of angiotensin I (ANG I) generated and then trapped by excess anti-ANG I antibody (Poulsen and Jorgensen 1973; Nussberger et al. 1987). In tubes coated with rabbit anti-ANG I antibody (Gamma Coat™ ^{125}I Plasma renin activity RIA kit; Baxter Travenol Diagnostics) and incubated in an ice-water bath, 75 µl plasma are mixed with 7 µl 3 M TRIS base buffer (pH 7.2) containing 200 mM EDTA, and 3 µl 0.2 M TRIS base (pH 7.5) containing 3 g/L human serum albumin (fraction V, Sigma). Tubes are vortexed and incubated at 37 °C for 60 min. The incubation is terminated by placing the tubes in an ice-water bath. Next, 75 µl of the TRIS albumin buffer are added, followed by 1 ml phosphate RIA buffer (Gamma Coat™) containing 15 000 cpm of ^{125}I ANG I. Standard ANG I (0.2–50 ng/ml) is also incubated at 37 °C for 60 min with 10 µl TRIS/albumin buffer. In an ice-water bath, low renin plasma (75 µl) is added to the standards before the addition of a 1 ml tracer solution. Samples and standards are incubated for 24 h at 4 °C. Tubes are then aspirated and counted in a gamma counter.

Levels of immunoreactive angiotensin II (ir-ANG II) are measured using a procedure described by Nussberger et al. (1985). Two–three ml of whole blood are collected in prechilled glass tubes containing 125 µl of the following "inhibitor" solution: 2% ethanol, 25 mM phenanthroline, 125 mM EDTA, 0.5 mM pepstatin A, 0.1 mM captopril, 2 g/l neomycin sulfate, and 0.1 mM of the renin inhibitor CGP 38560. The tubes are then centrifuged and the plasma quickly frozen in liquid nitrogen and stored at –70 °C.For extraction of angiotensin peptides, Bond-Elut cartridges (Bond-Elut-pH) containing 100 mg phenylsilica are used, along with a Vac Elut SPS24 vacuum manifold (Analytichem; Harbor City, CA). Each cartridge is preconditioned with 1.0 ml methanol (HPLC grade) followed by 1.0 ml of water (HPLC grade) at a vacuum pressure of 5 mm Hg. One ml of the thawed sample is then applied to the cartridge and washed with 3 ml HPLC grade water. The angiotensin peptides retained at the columns are eluted with 0.5 ml methanol (HPLC grade, vacuum pressure less than 5 mm Hg) into polypropylene tubes coated with a buffer containing 0.2 M TRIS, 0.02% NaN_3, and 2.5 mg/ml fatty acid-free bovine serum albumin (pH 7.4 with glacial ace-tic acid). The methanol is evaporated at 40 °C and ir-ANG II measured using an antibody (IgG Corp., Nashville, TN) with greater than 1 000-fold selectivity for ANG II.

EVALUATION

All data are expressed as mean ±SEM. The hypotensive responses after various doses of the renin antagonist are compared with the inhibition of plasma renin activity and the decrease of immunoreactive angiotensin II.

CRITICAL ASSESSMENT OF THE METHOD

The antibody-trapping method, reported here, gives a better correlation with the blood pressure lowering effect in dogs than the conventional method based on RIA for generated ANG I (Palmer et al. 1993).

MODIFICATIONS OF THE METHOD

Pals et al. (1990) described a rat model for evaluating inhibitors of human renin using anesthetized, nephrectomized, ganglion-blocked rats. The blood pressure rise induced by sustained infusion of renin was dose-dependently decreased by a renin inhibitor.

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A.1.3.34 Evaluation of renin inhibitors in monkeys

PURPOSE AND RATIONALE

The renin-angiotensin-system as the main regulator of blood pressure can be influenced in several ways. One approach involves the inhibition of renin. Renin is an aspartyl protease that hydrolyzes angiotensinogen to release the dekapeptide angiotensin I, which is subsequently converted to angiotensin II by angiotensin-converting-enzyme. Sequencing of renin and angiotensinogen from various species revealed marked species differences for both the enzyme and the substrate. Inhibitors developed for human renin show a high specificity for primate renin and show only weak inhibition of renin from sub-primate species. This means that the most common laboratory animals, such as rats and dogs, are not suitable for the *in vivo* evaluation of renin inhibitors. The marmoset was chosen by Wood et al. (1985, 1989) as a primate model.

PROCEDURE

Marmosets (*Callithrix jacchus*) of both sexes weighing between 300 and 400 g are fed a pellet diet supplemented with fruit. Two days prior to the experiment the animals are anesthetized and catheters are implanted in a femoral artery for measurement of blood pressure and in a lateral tail vein for injection or infusion of test substances. Thirty min before the experiment, the animals receive an intravenous injection of 5 mg/kg furosemide in order to stimulate renin release. During the experiment, the marmosets are sedated with diazepam (0.3 mg/kg i.p.) and kept in restraining boxes. Mean blood pressure is recorded continuously, and heart rate is measured at fixed intervals. The test compound or the standard are injected at various doses by intravenous infusion or administered orally.

EVALUATION

Blood pressure is recorded after 30 min of intravenous infusion and 30 min after stopping the infusion. Comparing the changes from pretreatment values after various doses, dose-response curves can be established.

MODIFICATIONS OF THE METHOD

Fischli et al. (1991) monitored arterial pressure in conscious and chronically instrumented monkeys using a telemetry system. One week before the experiment, the animals were anesthetized, and a 3F high fidelity pressure tip transducer (Millar Instruments, Inc.) was inserted into the abdominal aorta through the right femoral artery. Then the catheter was tunneled subcutaneously to the back of the monkey in the interscapular region. The proximal part of the catheter was connected to a transmitter located in a jacket worn by the monkey. The blood pressure was transmitted continuously to a receiver, which transformed the signal to an analogue value of blood pressure.

Linz et al. (1994) reported on the effects of renin inhibitors in anesthetized rhesus monkeys weighing between 5 and 13 kg. The animals are sodium-depleted by administration of 10 mg/kg/day furosemide-Na for 6 consecutive days. At day 7, 10 mg/kg furosemide is given i.v. 30 min before the start of the experiment. Anesthesia is induced with 20 mg/kg ketamine-hydrochloride i.m. and continued with 40 mg/kg pentobarbitone-Na, slow i.v. drip. After completion of surgical procedures and after insertion of catheters under fluoroscopic control, the following hemodynamic parameters are measured: Pulse rate, and systolic and diastolic blood pressures are registered with a transducer (Statham P23 ID) in one femoral artery. A catheter tip manometer (Millar Instruments, Houston, Texas, USA) is introduced into the left ventricular cavity for the determination of left ventricular pressure. Contractility is electronically deduced from left ventricular pressure with appropriate amplifiers (Hellige GmbH, Freiburg, Germany). The electrocardiogram (ECG) from conventional lead II is taken using an ECG transducer (Hellige GmbH). Heart rate is measured from QRS-peaks using a biotachometer (Hellige GmbH). Cardiac output is determined using the thermodilution method. Thermodilution is integrated and converted to cardiac output readings by commercially available equipment (HMV 7905, Hoyer, Bremen). To determine cardiac output, 2 ml chilled 0–5 °C isotonic glucose solution (5%) is injected rapidly into the right ventricle by a catheter via the right jugular vein. A thermistor is placed into the aortic arch via the right carotid artery.

Hemodynamics are monitored for 30 min following i.v. injection of various doses of the potential renin inhibitor. At the end of the experiments the ACE inhibitor ramiprilat 100 µg/kg is given i.v. to probe for an additional blood pressure lowering effect. Blood samples for the determination of ANG II concentration, renin inhibition and plasma drug levels are withdrawn at 10, 30 and 60 min after i.v. injection of the renin inhibitor. The volume is replaced by i.v. injections of isotonic glucose solution (5%). After all data and blood samples have been obtained, animals are sacrificed by an overdose of pentobarbitone-Na.

For experiments after intraduodenal administration sodium depletion and anesthesia are done as described above. A small side branch of the femoral or radial artery is surgically exposed and cannulated for blood pressure measurements using a pressure transducer (P23 ID). Heart rate is determined from a conventional ECG lead by a biotachometer. Blood samples are withdrawn via a catheter placed into the saphenous vein. A

gastric fiberscope (Olympus XP 10) is introduced into the duodenum under visual control and the renin inhibitor is administered intraduodenally through the service channel of the fiberscope in a volume of 5 ml. Blood samples are withdrawn before and at 15, 30, 45, 60, 90 and 120 min after intraduodenal administration.

CRITICAL ASSESSMENT OF THE METHOD

Due to the high species specificity of renin and its substrate, angiotensinogen, renin inhibitors for treatment of hypertension have to be tested in primate models. The marmoset as well as the rhesus monkey have been proven to be suitable models.

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A.1.4 Cardiovascular safety studies

A.1.4.1 General considerations

PURPOSE AND RATIONALE

The necessity to perform pharmacological tests in preclinical safety evaluation is obvious (Zbinden 1966; Alder and Zbinden 1973)

More recently, severe concern has been expressed on the cardiovascular safety of drugs. Some drugs have been reported to induce severe ventricular arrhythmias, including torsades de pointes, and have been responsible in some cases for sudden death of patients. Although the mechanisms of these arrhythmias are not well understood, they are often, but not always, associated with QT interval prolongation (Dessertenne et al. 1966; Motté et al. 1970; Jackman et al. 1988; Weissenburger et al. 1993; Cavero et al. 2000; Taglialatela et al. 2000). Regulatory agencies, such as the Committee for Propriety Medical Pruducts (CPMP), an arm of the European Agency for the Evaluation of Medicinal Products have pointed out the necessity to assess most carefully the potential for QT prolongation, especially for non-cardiovascular drugs (1997, 2000). *In vivo* studies, preferably in unrestrained animals, are requested.

The outcome of a Policy Conference on *The Potential of QT Prolongation and Pro-arrhythmia by Non-anti-arrhythmic Drugs. Clinical and Regulatory Implications* held in 1999 under the auspices of the European Society of Cardiology, Committee for Scientific and Clinical Initiatives, was published by Haverkamp et al. (2000). In this review not only the electrophysiological mechanisms of QT prolongation are discussed but also proposals for preclinical and clinical evaluations were made.

A concept paper is available on an ICH harmonized tripartite guideline on nonclinical studies for assessing risk of repolarization-associated ventricular tachyarrhythmias for human pharmaceuticals including advantages and limitations of the various models.

Eckardt et al. (1998) reviewed *in vitro* and *in vivo* experimental models of torsade de pointes.

Pourrias et al. (1999) proposed a three phase strategy for assessment of cardiac risk for non-cardiovascular drugs: first, telemetry in freely moving dogs, second, studying monophasic action potentials in anesthetized dogs under paced and nonpaced conditions, and finally, analysis by *in vitro* techniques, such as isolated Purkinje fibers. Lacroix and Provost (2000) proposed a similar strategy: First complete hemodynamic evaluation in anesthetized dogs, then electrical activity evaluation *in vivo* and *in vitro*, and finally autonomic nervous system evaluation as required by Japanese guidelines.

Another strategy using first *in vitro* and then *in vivo* preclinical models was proposed by Champeroux et al. (2000). A similar strategy was proposed by Gralinski (2000): First using *in vitro* single-cell techniques (channel blockade and action potential recordings), then isolated tissue models, followed by *in vivo* approaches to cardiac electrophysiology and *in vivo* models of pro-arrhythmia and finally clinical studies to examine potential for QT interval prolongation.

In this book, methods of cardiovascular analysis are described in A.1.3.1 to A.1.3.5 for rats, in A.1.3.6 for cats, in A.1.3.7 for mice, in A.1.3.8 to A.1.3.10 for dogs, and in A.1.3.11 for monkeys. Methods using animals chronically instrumented for telemetry are described in A.1.3.15.

Moreover, detailed studies on ECG *in vivo* are requested by the authorities for safety pharmacology. The methods for evaluation of ECG in various animal species are described in A.5.0.2.

Methods to detect arrhythmogenic properties of drugs are described below.

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A.1.4.2 Cardiovascular safety studies in dogs and other species

PURPOSE AND RATIONALE

Dogs are recommended as primary animal models to detect electrocardiographic changes, such as torsades de pointes, and effects of proarrhythmic drugs (Weissenburger et al. 1993; Salata et al. 1995; Matsunaga et al. 1998; Usui et al. 1998; Champeroux et al. 2000).

PROCEDURE

Mongrel dogs (8–12 kg) are anesthetized with 80–100 mg/kg i.v. α-chloralose and ventilated with a volume-cycled respirator. The right femoral artery and vein are isolated and cannulated for the measurement of systemic arterial pressure and for test agent administration, respectively. A left thoracotomy is performed in the fourth intercostal space, the percardium incised, and the heart suspended in a pericardial cradle. A platinum epicardial bipolar electrode is sutured to the left atrial appendage for atrial pacing, and a stainless steel bipolar plunge electrode is sutured to the anterior surface of the left ventricle for determination of ventricular excitation threshold and refractory periods. Limb electrodes are attached for continuous recording of the lead II electrocardiogram.

The following parameters are determined before and after cumulative intravenous administration of test agents: sinus heart rate, systemic arterial pressure, electrocardiographic intervals including a rate-corrected QTc interval according to Bazett's formula (Bazett 1920):

$$QTc = (QT\ [\text{ms}])\ (R - Rs)^{-1/2}$$

and a "paced QT" interval determined during 2.5-Hz atrial pacing, ventricular excitation threshold (VET), and ventricular effective refractory period (VERP, determined at two times ventricular excitation threshold). Groups of five to seven dogs are randomized to the intravenous administration of cumulative doses of test drugs. Each dose of test drug is administered over

5 min, with electrophysiological recording 15 min after termination of intravenous infusion.

Prolongation of QTc and ventricular effective refractory period indicate the risk for torsades de pointes.

EVALUATION

Data are expressed as mean ±SEM. Dunnett's *t*-test is used to determine significance of individual treatment means compared with control mean values. A multivariate repeated-measures ANOVA with comparison to the vehicle control group is used to identify significant within-group changes in electrocardiographic and electrophysiological parameters.

MODIFICATIONS OF THE METHOD

Champeroux et al. (2000) recommended the use of telemetric measurement of ECG in dogs for evaluation of the potential QT interval prolongation by drugs.

The use of Bazett's formula for heart rate correction of QT interval has been challenged. Sarma et al. (1984), Kovács (1985) proposed new exponential formulas to characterize the RR-QT relation in human beings.

Matsunaga et al. (1998) investigated the QT-RR relation in Beagle dogs by analysis of the QT and preceding RR intervals obtained from 24-hs ambulatory electrocardiograms. The acceptability of 14 QT prediction formulas was evaluated by use of 100–150 selected pairs of QT-RR points per animal in seven male and seven female Beagles. The accuracy of fit with the measured data was assessed. The one-parameter logarithmic formula

$$QTc = \log 600 \times QT / \log RR$$

was proposed for correcting the QT interval at a heart rate of 100 beats/min.

Champeroux et al. (2000) calculated the a, b and c parameters of Sarma's formula:

$$QT = a\, e^{(b\,RR)} + c$$

by fitting the QT/RR relationship from pairs of QT/RR values measured by telemetry from control dogs.

Hey et al. (1996) analyzed the ECG wave in anesthetized **guinea pigs** to determine QT interval, QTc interval, PR interval QRS interval and heart rate after the administration of the second generation antihistamines ebastine and terfenadine. In separate studies in conscious guinea pigs, the effect of oral ketoconazole on the ECG parameters after oral ebastine and terfenadine was studied.

Carlsson et al. (1990, 1993, 1997) studied class III effects and proarrhythmic properties of drugs in **rabbits** anesthetized with methohexital-sodium and α-chloralose. After base-line measurements of ECG, arterial blood pressure and heart rate, a continuous infusion of the α_1-agonist methoxamine (70 nmol/kg/min) was started. Ten min later, test drugs or vehicle were continuously infused for 10 min and the ECG was continuously monitored. The ECG analysis was subsequently performed on averaged ECG complexes. The T wave usually has a bifid appearance in the rabbit (TU complex). The QTU interval was defined as the time between the first deviation from the isoelectric line during the PR interval and the second peak of the TU complex. Torsades de pointes was defined as a transient tachyarrhythmia seen in the presence of QTU interval lengthening, with a typical initiation ("short-long-short sequence) and more than five consecutive undulating peaks of sequential QRS com-plexes observed in at least two leads.

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A.1.4.3 Monophasic action potentials in dogs

PURPOSE AND RATIONALE

Registration of monophasic action potential (MAP) in anesthetized dogs for evaluation of arrhythmogenic activity of drugs was recommended by Usui et al. (1998), Weissenburger et al. (2000).

PROCEDURE

Beagle dogs of either sex are initially anesthetized with 30 mg/kg i.v. thiopental sodium. After intubation, 1% halothane is inhaled with a volume-limited ventilator. Systemic blood pressure is measured at the right femoral artery. An ECG is obtained from the limb electrodes. A thermodilution catheter is inserted through the right femoral vein and positioned at the right side of the heart. Cardiac output is measured by a standard thermodilution method using a cardiac output computer. A pig-tail catheter is inserted through the right femoral artery and positioned at the left ventricle to measure the left ventricular pressure. The maximum upstroke velocity of the left ventricular pressure (LV dP/dt) and the left ventricular end-diastolic pressure (LVEDP) are measured to estimate the contractility and the preload of the ventricle, respectively.

A quad-polar electrode catheter is inserted through the left femoral artery and positioned at the non-coronary cup of the aortic valve to record a His bundle electrogram. A bi-directional steerable monophasic action potential (MAP) recording/pacing combination catheter is inserted through the left femoral vein and positioned at the endocardium to obtain MAP signals. The signals are amplified with a DC amplifier.

The amplitude of MAP is measured as the distance from the diastolic baseline to the crest of the MAP plateau phase. The duration of the MAP signal is measured as an interval from the MAP upstroke to the desired repolarization level along a line horizontal to the diastolic baseline. The interval [ms] at 90% repolarization is defined as MAP_{90}. The heart is driven electrically through the pacing electrodes of the combination catheter. Stimulation pulses are rectangular in shape, about twice the threshold voltage (1.5–2.2 V) and of 1 ms duration. The MAP_{90} is measured during sinus rhythm (MAP_{90}(sinus)), and at a pacing cycle length of 400 ms (MAP_{90}(400)) and 300 ms (MAP_{90}(300)).

The effective refractory period (ERP) of the right ventricle is assessed with a programmed electrical stimulator. The pacing protocol consists of 8 beats of basal stimuli in a cycle length of 400 ms followed by an extra stimulus of various coupling intervals. Starting in late diastole, the coupling interval is shortened by 5–10 ms steps until refractoriness occurs. The difference ERP – MAP_{90}(400) is calculated to predict the vulnerability of the myocardium.

The amplified MAP signals together with systemic blood pressure, left ventricular pressure, heart rate and ECG are continuously monitored using a polygraph system. Each value of ECG and MAP represents the mean of three consecutive complexes. Corrected QT interval (QTc) is obtained using the formula of Bazett (1920).

EVALUATION

Data are presented as mean ± SM. The statistical significance of the effect of tested drugs on each parameter is evaluated by one-way repeated-measured analysis of variance (ANOVA). When a P-value is less than 0.05 by ANOVA, the statistical significance between a group is determined by a computer program, Contrast for mean values comparison. The differences of the control values between the groups is analyzed by a paired t-test.

MODIFICATIONS OF THE METHOD

Chen et al. (1999), Hsieh et al. (2000) developed a canine model of pacing induced cardiomyopathy to study the possible mechanisms of drug-induced torsades de pointes. Twelve-lead ECG and right and left ventricle monophasic action potentials were recorded at different right ventricle pacing cycle lengths from 600 ms to 1 200 ms.

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A.1.4.4 Studies of arrhythmogenic effects in isolated heart preparations

PURPOSE AND RATIONALE

The evaluation of arrhythmogenic, anti-arrhythmic and antifibrillatory effects in the LANGENDORFF heart preparation is described in Sect. A.3.1.2.

Eckardt et al. (1998), Johna et al. (1998) proposed the isolated perfused rabbit heart as a model to study proarrhythmia induced by class III antiarrhythmic drugs.

PROCEDURE

Male New Zealand white rabbits weighing 2.5–3.0 kg are anti-coagulated with heparin sodium (2000 IU) and anesthetized with thiopental sodium via a marginal ear vein. After transecting the carotid arteries, the chest is opened and the heart rapidly excised. A cannula is inserted into the aortic stump, the pulmonary artery incised, and the spontaneously beating heart perfused through the coronary arteries at 37 °C in a non-circulating system at a constant pressure of 70 mm Hg with Krebs-Henseleit buffer containing (in mM): $CaCl_2$ 2.52; KCl 4.70; KH_2PO_4 1.18; $MgSO_4$ 1.66; NaCl 118; $NaHCO_3$ 24.88, Na-pyruvate 2.00; glucose 5.55 (= solution I). The buffer is gassed with 95% O_2 and 5% CO_2 yielding a pH of 7.4. Solution II contains KCl for a total of 2.0 mM K^+ and Mg^{2+} 0.5 mM; the other components are left unchanged. The heart is immersed in an organ bath at 37 °C. The coronary flow is estimated by collecting the outflow of the bath in a bowl placed on an electronic balance from which the values are read out by a computer at 10 s intervals. Electrocardiograms are touchlessly recorded from the bath via Ag/AgCl electrodes (filter settings: 0.1–300 Hz) in two perpendicular directions in the horizontal plane. QT intervals are measured from the beginning of the QPS complex to the intersection of the descending part of the T wave with the baseline in either ECG lead. The larger value is used for further analysis. The corrected QT (QTc) is calculated using Bazett's formula. Monophasic action potentials (MAP) are obtained using a commercially available Franz combination catheter (Franz 1994) (EP Technologies Inc., Sunnyvale, CA, USA) placed epicardially on the left ventricular posterior wall. Isovolumetric left ventricular pressure (LVP) is measured via a pressure transducer connected to a water-filled latex balloon attached to a rigid catheter and inserted through the left atrium into the ventricle (preload 5–10 mm Hg). ECGs, MAPs, and LVP are registered with a strip chart recorder, digitized at a rate of 1 kHz with 12 bit resolution and stored on discs for further analysis. Drugs are infused through an injection port above the aorta.

After a baseline period of 10 min, the test drug or a standard drug (Clofilium, a class III antiarrhythmic drug, Steinberg and Molloy 1979; Friedrichs et al. 1994) is infused for 20 min and MAP recordings are started. Following 5 more min of solution I (K^+ 5.88 mM, Mg^{2+} 1.66 mM) the perfusion is switched to solution II (K^+ 2.0 mM, Mg^{2+} 0.5 mM). During the subsequent 5 min the cardiac rhythm is described in consecutive 5 s windows by means of the ECG. The following patterns are distinguished: normal sinus rhythm, bigeminal pattern, monomorphic ventricular activity, ventricular rhythm with two alternating QRS morphologies and polymorphic ventricular tachyarrhythmias of the TdP type with undulating peaks of sequential QRS complexes (Jackman et al. 1988). MAP durations are measured at the 50%, 70% and 90% repolarization level. Subsequently, the hearts are again perfused with solution I.

EVALUATION

All values are expressed as mean ±SD. Statistical evaluation is carried out by two-tailed *t*-test for paired or unpaired observations.

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A.1.4.5 Studies in isolated Purkinje fibres

PURPOSE AND RATIONALE

Studies in isolated rabbit Purkinje fibers were recommended to assess the risk of QT interval prolongation by drugs (Adamantidis et al. 1995, 1998; Cavero et al. 1999; Dumotier et al. 1999; Champeroux et al. 2000).

PROCEDURE

New Zealand white rabbits of either sex weighing 1.5–2 kg are sacrificed by cervical, dislocation and exsanguinated. Their hearts are quickly excised and placed in potassium- and glucose-enriched Tyrode solution (in mM: NaCl 108.2; KCl 27; $CaCl_2$ 1.8; $MgCl_2$ 1; NaH_2PO_4 1.8; $NaHCO_3$ 25; glucose 55; pH 7.35 ±0.05)

oxygenated (95% O_2/5% CO_2) at a temperature of about 32 °C. The left ventricle is opened through an incision into the anterior intraventricular groove. Purkinje fibers still attached to the ventricular muscle are carefully dissected and pinned to the silicone base of the experimental chamber. The pins are exclusively placed in ventricular muscle to avoid stretching of the Purkinje fibers. The preparations are superfused for 30 min, at a flow rate of 2 ml/min with the above-described solution maintained at 36.5 ±0.5 °C. Then the superfusate is switched to normal Tyrode's solution (in mM: NaCl 108.2; KCl 4; $CaCl_2$ 1.8; $MgCl_2$ 1; NaH_2PO_4 1.8; $NaHCO_3$ 25; glucose 11). The preparations are electrically stimulated by rectangular pulses of 1 ms with a frequency of 120 pulses per min and an intensity 1.5 times the diastolic threshold. After 30 min at least, the stimulation frequency is turned to 60 pulses per min.

Transmembrane action potentials are recorded using 3M KCl glass microelectrodes with a tip resistance of 10–15 megohms, which are coupled with an Ag-AgCl bath electrode and connected to an impedance amplifier, and are displayed on an oscilloscope with numerical memory, analyzed by an external computer system and stored on a digital magnetic type recorder which allows display on paper recordings of the occurrence and development of electrical abnormalities. The following parameters are measured: testing membrane potential, action potential and overshoot, maximal rate of depolarization and action potential duration at 50% and 90% of repolarization.

The studied drug is added to the Tyrode's solution in increasing cumulative concentrations, each concentration being tested for 30 min. The stimulation frequency is 60 pulses per min except between the 20th and 22nd min, when the frequency is reduced to 12 and then switched bach to 60 pulses per min. Action potentials are measured after 15 and 30 min of each concentration superfusion and at the end of the 2-min stimulation of 12 pulses per min.

EVALUATION

The results are expressed as means ±SEM. Comparisons vs. controls are performed statistically using an analysis of variance for repeated measures completed by the corrected Dunnett's *t*-est.

MODIFICATIONS OF THE METHOD

Kondo et al. (1999) studied the antagonism of potassium channel openers against the effect of class III antiarrhythmic agents in Purkinje fibers of **dogs**.

Studenik et al. (1999) used isolated spontaneously beating Purkinje fibers from **guinea pigs** to study the proarrhythmic potential of antidepressant and neuroleptic drugs.

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A.1.4.6 Studies in isolated ventricular myocytes

PURPOSE AND RATIONALE

Analysis of action potential and patch-clamp techniques in isolated ventricular myocytes are used to clarify the mechanisms underlying the development of torsade de pointes ventricular arrhythmias (Salata et al. 1995; Drolet et al. 1999; Kalifa et al. 1999).

PROCEDURE

Preparation of single cells

Guinea pigs weighing 250–350 g are sacrificed by decapitation and their hearts quickly removed (Jurkiewicz et al. 1993). They are perfused retrogradely through the aorta at a rate of 10 ml/min with an oxygenated Ca^{2+}-free HEPES-buffered saline (containing 132 mM NaCl, 4 mM KCl, 1.2 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.2) at 37 °C for 5 min, then with the same solution containing 300 U/ml type II collagenase and 0.5 to 1.0 U/ml type XIV protease for 8 min, and finally with free HEPES-buffered saline containing 0.2 mM $CaCl_2$ for an additional 5 min. The enzyme-digested heart is cut into small pieces, placed in 20 ml HEPES-buffered saline containing 0.2 mM $CaCl_2$ and

shaken until single cells are dissociated from the small pieces. The cells are then filtered through a 200-μm nylon mesh and resuspended in HEPES-buffered saline containing 1.8 mM $CaCl_2$ and stored at 24 °C until use.

Action potential studies

Transmembrane potentials are recorded using conventional glass electrodes filled with 3 M KCl (tip resistances, 35–60 MΩ). They are connected to the headstage of an Axoclamp 2A amplifier (Axon Instruments. Cells are superfused with HEPES-buffered saline containing 1.8 mM $CaCl_2$ at a rate of 2 ml/min at 37 °C. Action potentials are evoked by passing brief current pulses (1 ms, 1.2 times threshold) through the recording electrode using an active bridge circuit. Cells are stimulated at a frequency of 1 Hz during a 10–15-min stabilization period before control measurements are obtained. Action potential are studied at frequencies of 1 and 3 Hz during control and at 10 min after superfusion with test agents at cumulatively increasing drug concentrations. Individual action potentials are sampled after 10-s trains of stimuli at each frequency, and four samples are digitally averaged and then measured for each condition.

Voltage-clamp studies

Microelectrodes made from square-bore (1.0 mm outer diameter) borosilicate capillary tubing are filled with 0.5 M K^+ gluconate, 25 mM KCl, 5 mM K_2ATP having a resistance of 3–7 MΩ (Sanguinetti and Jurkiewicz 1990). A List EPC-7 clamp amplifier is used to voltage clamp the isolated cells. Series resistance is compensated 40% to 70%, and current is low-pass filtered at a cutoff frequency of 1 kHz.

Voltage clamp is performed using the whole-cell recording mode, and cell perfusion is minimized by maintaining constant negative pressure on the electrode using a 1-ml gas-tight syringe attached to the suction port of the microelectrode holder via air-tight tubing.

Outward potassium currents are measured during superfusion of the cells at a rate of 2 ml/min with Ca^{2+}-free HEPES-buffered saline (35 °C) containing 0.4 μM nisoldipine to block L-type Ca^{2+} current (I_{Ca}). Cells are voltage-clamped at a holding potential (V_h) of either −50 or −40 mV to inactivate inward Na^+ current (I_{Na}). The "steady-state" current-voltage (I–V) relation for K^+ currents is examined initially by using a slowly depolarizing voltage ramp beginning at −110 mV and ending at +50 mV at a rate of 32 mV/s. I_K is measured as the difference from the initial instantaneous current, following the settling of the capacity transient, to the final (steady-state) current level during depolarization voltage steps to various test potentials (V_t). Tail current amplitude (I_{Ktail}) is measured as the difference from the holding current level to the peak I_{Ktail} on return to V_h. Inward rectifier K^+ current (I_{Ki}) is measured as the absolute current, uncorrected for leak, either during depolarizing voltage ramps or at the end of 225-ms hyperpolarizing voltage steps from a V_h of −40 mV.

Concentration-response relations are determined by measuring action potentials of currents in each cell during control conditions and during superfusion with two successively increasing concentrations of a given drug. Concentration-response curves are fit to a logistic equation, $Y = (a - d) / [1 + (X / c)^b] + d$, using a Marquardt-Levenburg algorithm for least-squares nonlinear regression analysis. Using this equation, a and d are maximal and minimal responses estimated for infinite and zero concentrations, respectively; c is the inflection point that estimates the 50% effective concentration (IC_{50}); and b is the slope factor (Hill coefficient). During the time required (4 to 8 min) to achieve steady-state drug effects, I_{Ktail} measured after a voltage step from −50 to −10 mV, which is used to quantify I_{Kr}, decreases by an average of 5% even in the absence of drug or vehicle. Because of this "rundown" of the current the concentration-response curves are fit with a minimal inhibition of 5%.

EVALUATION

Data are expressed as mean ±SEM. Action potentials are assessed using a three-way ANOVA to determine significant within-treatment variations. Dunnett's t-test is used to determine significance of individual treatment means compared with control mean values. A multivariate repeated-measures ANOVA with comparison to the vehicle control group is used to identify significant within-group changes in electrophysiological parameters.

MODIFICATIONS OF THE METHOD

Carlsson et al. (1997) performed voltage clamp studies in isolated ventricular myocytes from **rabbits**.

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A.2 Methods to induce experimental hypertension

A.2.0.1 Acute renal hypertension in rats

PURPOSE AND RATIONALE

Since the classical experiments of Goldblatt et al. (1934) there is clear evidence that the ischemia of the kidneys causes elevation of blood pressure by activation of the renin-angiotensin system. The principle can be used both for acute and chronic hypertension. In rats acute renal hypertension is induced by clamping the left renal artery for 4 h. After reopening of the vessel, accumulated renin is released into circulation. The protease renin catalyzes the first and rate-limiting step in the formation of angiotensin II leading to acute hypertension. The test is used to evaluate antihypertensive activities of drugs.

PROCEDURE

Male Sprague-Dawley rats weighing 300 g are used. The animals are anesthetized by intraperitoneal injection of 100 mg/kg hexobarbital sodium. A PVC-coated Dieffenbach clip is placed onto the left hilum of the kidney and fixed to the back muscles. The renal artery is occluded for 3.5–4 h.

3.5 h following the surgery, the animals are anesthetized by intraperitoneal injection of 30–40 mg/kg pentobarbital sodium. The trachea is cannulated to facilitate spontaneous respiration. To measure systolic and diastolic blood pressure, the cannula in the carotid artery is connected to a pressure transducer (Statham P 23 Db).

For administration of the test compound, a jugular vein is cannulated.

Following a stable blood pressure state, ganglionic blockade is performed with pentolinium (10 mg/kg i.v.). After obtaining stable reduced blood pressure values, the renal arterial clip is removed. This leads to a rise in blood pressure as a consequence of elevated plasma renin level. Within 15 min a stable hypertension is achieved (control = 100%).

The test substance is then administered by intravenous injection at doses of 10 and 100 µg/kg.

Blood pressure is monitored continuously until a renewed increase to the starting level is obtained.

Ten–twelve animals are used per compound.

EVALUATION

Increase in blood pressure after reopening of the renal artery and reduction in blood pressure after administration of the test drug are determined [mm Hg]. Percent inhibition of hypertensive blood pressure values under drug treatment are calculated as compared to pretreatment hypertension values. Duration of the effect is determined [min]. Statistical significance is assessed by the paired *t*-test.

MODIFICATIONS OF THE METHOD

A sharp and transient in systemic arterial blood pressure associated with reflex bradycardia can be elicited by injection of 5-hydroxytryptamine, cyanide, nicotine or lobeline into the coronary artery blood stream of dogs (Berthold et al. 1989). The phenomenon is named the cardiogenic hypertensive chemoreflex and 5-HT proved to be the most powerful agent for its initiation (James et al. 1975).

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A.2.0.2 Chronic renal hypertension in rats

PURPOSE AND RATIONALE

On the basis of the findings of Goldblatt et al. (1934) that ischemia of the kidneys induces hypertension, various modifications of the technique have been described for several animal species. One of the most effective modifications in rats is the so called 1-kidney-1-clip method.

PROCEDURE

Male Sprague-Dawley rats weighing 200–250 g are anaesthetized with 50 mg/kg i.p. pentobarbital. The fur on the back is shaved and the skin disinfected. In the left lumbar area a flank incision is made parallel to the long axis of the rat. The renal pedicel is exposed with the kidney retracted to the abdomen. The renal artery is dissected clean and a U-shaped silver clip is slipped around it near the aorta. Using a special forceps (Schaffenburg 1959) the size of the clip is adjusted so that the internal gap ranges from 0.25–0.38 mm. The right kidney is removed through a flank incision after tying off the renal pedicle. The skin incisions are closed by wound clips.

4–5 weeks after clipping blood pressure is measured and rats with values higher than 150 mm Hg selected for the experiments. Blood pressure readings are taken on each of 3 days prior to drug treatment. Drugs are administered orally in volumes of 10 ml/kg. The rats are divided into 4 animals per dose and each animal is used as his own control. Compounds are administered for 3 days and predrug and 2 h postdrug blood pressure readings are taken.

EVALUATION

Changes in systolic blood pressure are expressed in mm Hg. Activity is determined by comparing treatment blood pressure values with the control blood pressure value (Day 1, predrug blood pressure). Comparisons are made using the paired *t*-test for evaluation of statistical significance.

MODIFICATIONS OF THE METHOD

Duan et al. (1996) induced renal hypertension in male Hartley **guinea pigs** by a two-step procedure consisting of ligation of the left caudal renal artery and right nephrectomy. Arterial blood pressure and heart rate were monitored in conscious animals. ACE-inhibitors reduced blood pressure in sham-operated and in renal hypertensive guinea pigs, whereas renin inhibitors were effective only in renal hypertensive animals.

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A.2.0.3 Chronic renal hypertension in dogs

PURPOSE AND RATIONALE

Production of hypertension by clamping renal arteries has been first described by Goldblatt et al. (1934) in dogs. Later on, the method has been modified, e.g., as the "wrapping" technique (Abram and Sobin 1947).

PROCEDURE

Dogs weighing 8–12 kg are anesthetized with i.v. injection of 15 mg/kg thiopental. Anesthesia is maintained with a halothane-oxygen mixture. Under aseptic conditions, a midline abdominal incision is made. One kidney is exposed and wrapped in cellophane and then replaced. The contralateral kidney is exposed. The artery, vein and ureter are ligated and the kidney is removed. The abdomen is closed by sutures and clips. On the day of surgery and for 3 days following, the dogs are given antibiotics. Body temperature is measured twice daily for 4 days following surgery.

Six weeks following surgery, blood pressure is measured using a tail-cuff method. For recording, the tail-cuff is attached to a polygraph. Only animals with a systolic blood pressure higher than 150 mm Hg are considered to be hypertensive and can participate in studies evaluating potential antihypertensive compounds.

For the experiment, blood pressure is recorded either by the indirect tail-cuff method or by direct measurement via an implanted arterial cannula. On day 1 readings are made every 2 h, just before, and 2 and 4 h after oral treatment with the potential antihypertensive compound. Drug administration is repeated for 5 days. On days 3 and 5 blood pressure readings are taken before and 2 and 4 h after treatment. At least 3 dogs are used per dose and compound.

EVALUATION

The starting value is the average of the 2 readings before application of the drug. Each of the following readings is subtracted from this value and recorded as fall of blood pressure at the various recording times.

MODIFICATIONS OF THE METHOD

Renal hypertension in rats has been achieved by many modifications of the method (Stanton 1971) such as

the technique according to Grollman (1944). The kidney is exposed through a lumbar incision, the renal capsule is removed by gentle traction, and a figure-8 ligature is applied being tight enough to deform the kidney but not tight enough to cut the tissue.

Renal hypertension may be induced in the **rat** by encapsulating both kidneys with latex rubber capsules (Abrams and Sobin 1947). Moulds are formed from plastic using a rat kidney as a model. The capsules are prepared by dipping the moulds in liquid latex allowing them to dry in the air. Three applications of latex are applied before the capsules are toughened by placing them under warm running tap water. The kidney is exposed by lumbar incision, the renal capsule gently removed and the capsule applied.

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A.2.0.4 Neurogenic hypertension in dogs

PURPOSE AND RATIONALE

Vasodilator and depressor reflexes, originating in the baroreceptor areas of the carotid sinus and aortic arch, play an important part in the regulation of blood pressure. Stimulation of the afferent buffer fibres exerts an inhibitory influence on the vasomotor center, and their sectioning leads to a persistent rise in blood pressure. In this way, acute neurogenic hypertension can be induced in dogs.

PROCEDURE

Adult dogs of either sex weighing 10–15 kg are anesthetized using 15 mg/kg sodium thiopental, 200 mg/kg sodium barbital and 60 mg/kg sodium pentobarbital i.v. A femoral vein and artery are cannulated using polyethylene tubing to administer compounds i.v. and record arterial pressure and heart rate, respectively. Left ventricular pressure and *dp*/*dt* are recorded via the left common carotid artery (post-deafferentiation) using a Millar microtip pressure transducer. P_{max} is recorded by speeding up the chart paper. Cardiac output is determined by introducing a Swan-Ganz catheter into the right heart and pulmonary artery via a jugular vein. Five ml of cold 5% dextrose is injected into the right atrium and an Edwards Cardiac output computer is used to calculate the cardiac output from the temperature change in the pulmonary artery. All recordings are made with a polygraph.

Both of the carotid arteries are cleared up to the bifurcation of the internal and external carotid arteries. The carotid sinus nerves are isolated, ligated and sectioned and a bilateral vagotomy is performed to produce neurogenic hypertension (mean arterial pressure more than 150 mm Hg). The dog is allowed to equilibrate for approximately 30 min and a bolus of the test compound is administered by intravenous injection. Heart rate, arterial pressure, left ventricular pressure, *P*max and *dp*/*dt* are monitored for 90 min. A minimum of 3 dogs are used for each compound.

EVALUATION

Changes of the cardiovascular parameters are expressed as percentage of the values before administration of the drug.

MODIFICATIONS OF THE METHOD

Neurogenic hypertension through baroreceptor denervation has also been described in **rabbits** (Angell-James 1984) and in rats (Krieger 1984).

CRITICAL ASSESSMENT OF THE METHOD

The neurogenic hypertension is useful for acute experiments. However, it is less useful for chronic experiments since the elevated blood pressure caused by buffer nerve section is more labile than that caused by renal ischemia.

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A.2.0.5 DOCA-salt induced hypertension in rats

PURPOSE AND RATIONALE

Mineralocorticoid-induced hypertension is thought to be due to the sodium retaining properties of the steroid causing increases in plasma and extracellular volume. The hypertensive effect is increased by salt loading and unilateral nephrectomy in rats.

PROCEDURE

Male Sprague Dawley rats weighing 250–300 g are anesthetized with ether. Through a flank incision the left kidney is removed. The rats are injected twice weekly with 20 mg/kg s.c. desoxycorticosterone-acetate in olive oil for 4 weeks. Drinking water is replaced with a 1% NaCl solution. Blood pressure starts to rise after one week and reaches systolic values between 160 and 180 mm Hg after 4 weeks.

MODIFICATIONS OF THE METHOD

The regimen to induce DOCA-salt hypertension has been modified by many authors (Stanton 1971).

DOCA pellets (Peterfalvi and Jequier 1960; Passmore and Jimenez 1990) or implants in silastic devices (Ormsbee and Ryan 1973; King and Webb 1988) were used instead of repeated injections.

DOCA-salt hypertension can also be achieved without nephrectomy (Bockman et al. 1992).

Using kininogen-deficient Brown Norway Katholiek (BN-Ka) rats, Majima et al. (1991, 1993) showed suppression of rat desoxycorticosterone-salt hypertension by the kallikrein-kinin system.

Li et al. (1996) examined small-artery structure on a wire myograph and quantified endothelin-1 messenger RNA by Northern blot analysis in DOCA-salt hypertensive rats after administration of an ACE-inhibitor, a calcium channel antagonist and a nitric oxide synthase inhibitor.

Ullian (1997) described the **Wistar-Furth rat** as a model of mineralocorticoid resistance. These rats developed two-kidney, one-clip hypertension to the same degree as did Wistar rats and reacted to glucocorticoid treatment with a rapid onset of hypertension, but were resistant to the development of DOCA-NaCl hypertension.

Studies in DOCA-salt hypertensive **mice** were reported by Gross et al. (1998, 1999), Honeck et al. (2000), Peng et al. (2001).

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A.2.0.6
Fructose induced hypertension in rats

PURPOSE AND RATIONALE

Increases in dietary carbohydrate intake can raise blood pressure in experimental animals. The increased intake of either sucrose or glucose was shown to enhance the development of either spontaneous hypertension or salt hypertension in rats (Hall and Hall 1966; Preuss and Preuss 1980; Young and Landsberg 1981). Hwang et al. (1987) first reported that hypertension could be induced in normal rats by feeding a high-fructose diet. Fructose feeding was also found to cause insulin resistance, hyperinsulinemia, and hypertriglyceridemia in normal rats (Zavaroni et al. 1980; Tobey et al. 1982). Dai and McNeill (1995) studied the concentration- and duration-dependence of fructose-induced hypertension in rats.

PROCEDURE

Groups of 8 male Wistar rats weighing 210–250 g are used. The are housed two per cage on a 12-h light 12-h dark cycle and are allowed free access to standard laboratory diet (Purina rat chow) and drinking fluid. Drinking fluid consists either of tap water or 10%-fructose solution. Body weight, food intake and fluid intake of each rat are measured every week during treatment. Using the tail-cuff method, systolic blood pressure and pulse rate is measured before and every week during treatment. Blood samples are collected before and every second week during treatment for determination of plasma glucose, insulin, and triglycerides.

EVALUATION

Since maximum effects on the chosen parameters are achieved after 6 weeks, the duration of treatment can be limited to this time. Statistical analysis is performed using a one-way or two-way analysis of variance, followed by the Newman-Keuls test.

MODIFICATIONS OF THE METHOD

Reaven et al. (1988, 1989) found an attenuation of fructose-induced hypertension by exercise training and an inhibition by somatostatin treatment.

Brands et al. (1991, 1992) found an increase of arterial pressure during chronic hyperinsulinemia in conscious rats.

Hall et al. (1995) reported the effects of 6 weeks of a high-fat diet on cardiovascular, renal, and endocrine functions in chronically instrumented conscious **dogs**. Body weight increased by approximately 16.9 kg, whereas MAP, cardiac output, and heart rate increased by 28%, 77%, and 68%, respectively.

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A.2.0.7
Genetic hypertension in rats

SURVEY

Inherited hypertension in rats has been described by Smik and Hall 1958; Phelan and Smirk 1960; Laverty and Smirk 1961; Phelan 1968 as **genetically hypertensive (GH) rats** (Simpson and Phelan 1984).

Okamoto et al. (1963, 1966) reported the development of a strain of spontaneously hypertensive rats from mating one Wistar male rat with spontaneously occurring high blood pressure with a female with slightly elevated blood pressure. By inbreeding over several generations a high incidence of hypertension with blood pressure values of 200 mm Hg or more was achieved. These strains were called "**Spontaneously hyperten-**

sive rats (Akamoto-Aoki)" = SHR or **"Wistar-Kyoto rats" = WKY**. Hypertension in these rats is clearly hereditary and genetically determined, thus comparable to primary hypertension in humans. Cardiac hypertrophy (Sen et al. 1974) and cellular ionic transport abnormalities have been observed (Yamori 1984).

Inbred strains being **salt-hypertension-sensitive (DS)** and **salt-hypertension-resistant (RD)** have been developed by Dahl et al. (1962, 1963), Rapp (1984), Cicila et al. (1993).

Two strains of rats with inbred dissimilar sensitivity to DOCA-salt hypertension (**"Sabra strain", SBH, SBN**) have been separated by Ben-Ishay et al. (1972, 1984).

Another hypertensive strain derived from Wistar rats was produced by brother-sister mating in the group of Bianchi et al. (1974, 1986) at the University of Milan called **"Milan hypertensive strain" = MHS**. These rats show a cell membrane defect resulting in abnormal kidney function. Salvati et al. (1990) studied the diuretic effect of bumetanide in isolated perfused kidneys of Milan hypertensive rats.

Furthermore, the ***"Lyon"*** strains of hypertensive, normotensive and low-blood-pressure rats were developed (Dupont et al. 1973; Vincent et al. 1984; Dubay et al. 1993). These rats show a genetically determined defect in central nervous function.

Several substrains of spontaneous hypertensive rats were separated by the group of Okamoto et al. (1974) including the **stroke-prone strain SHR = SHRSP**. These rats have an increased sympathetic tone and show a high incidence of hemorrhagic lesions of the brain with motor disturbances followed by death (Yamori 1984; Feron et al. 1996).

A strain of obese spontaneously hypertensive rats has been described by Koletsky (1975), Ernsberger et al. (1993).

Increase of blood pressure of spontaneously hypertensive rat is determined by multiple genetic loci (Deng and Rapp 1992; Dubay et al. 1993). With new technology not only these loci can be defined but also with **transgenic animals** new models in hypertension research and models to detect antihypertensive drugs can be established. A newly established **rat strain TGR(mREN2)27** as a monogenetic model in hypertension research was described by Peters et al. (1993).

CRITICAL ASSESSMENT OF THE METHOD

The use of spontaneously hypertensive rats to detect potential antihypertensive compounds is well established. On the basis of available data no preference can be given to a particular strain. The most abundant experience has been gained with the Wistar-Kyoto strain. Transgenic rats with well defined genomes are gaining more importance.

MODIFICATIONS OF THE METHOD

Pijl et al. (1994) described streptozotocin-induced diabetes mellitus in spontaneously hypertensive rats as a pathophysiological model for the combined effects of hypertension and diabetes.

Rosenthal et al. (1997) used rats of the Cohen-Rosenthal diabetic hypertensive strain to examine the effects of an ACE-inhibitor, an ATII antagonist and a calcium antagonist on systolic pressure and spontaneous blood glucose levels.

Linz et al. (1997) compared the outcome of lifelong treatment with the ACE inhibitor ramipril in young prehypertensive stroke-prone spontaneously hypertensive rats and age-matched normotensive Wistar-Kyoto rats. Lifelong ACE inhibition doubled the lifespan in hypertensive rats matching that of normotensive rats.

Studies in **genetically hypertensive mice** were reported by Rosenberg et al. (1985), Hamet et al. (1990) Meneton et al. (2000).

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A.2.0.8 Pulmonary hypertension induced by monocrotaline

PURPOSE AND RATIONALE

The pyrrolizidine alkaloid monocrotaline, derived from *Crotalaria spectabilis*, is hepatotoxic and pneumotoxic in the rat. A single injection of monocrotaline leads to progressive pulmonary hypertension resulting in right ventricular hypertrophy and cardiac failure (Gillespie et al. 1986, 1988; Todorovich-Hunter et al. 1988). Pathologic changes and hemodynamic changes associated with monocrotaline administration include blebbing of the lung, degeneration and fragmentation of endothelial cells, perivascular edema, extravasation of red blood cells, and muscularization of the pulmonary arteries and arterioles (Valdiva et al. 1967; Lalich et al. 1977; Huxtable et al. 1978; Hislop and Reid 1979; Meyrick and Reid 1979; Meyrick et al. 1980; Ghodsi and Will 1981; Hilliker et al. 1982; Sugita et al. 1983; Hilliker and Roth 1985; Stenmark et al. 1985; Altieri et al. 1986; Molteni et al. 1986; Lai et al. 1996). Rats given monocrotaline develop severe right ventricular hypertrophy often accompanied by ascites and pleural effusions (Ceconi et al. 1989).

Amelioration by angiotensin-converting enzyme inhibitors and by penicillamine has been demonstrated (Molteni et al. 1985, 1986).

PROCEDURE

Treatment of male Sprague Dawley rats weighing 200–225 g with the test drug (angiotensin converting enzyme inhibitor or vehicle) is started one week prior to a single subcutaneous injection of 100 mg/kg monocrotaline up to sacrifice 4, 7, or 14 days later by pentobarbital anesthesia and exsanguination. Heart and lungs are excised from thoracic cavity. After removing atria from the heart, the right ventricle is separated from the left ventricle plus septum which are blotted and weighed separately. Left lung is blotted, weighed, minced and reweighed after drying at room temperature for 14 days. Three pulmonary artery segments, main pulmonary artery, right extrapulmonary artery and an intrapulmonary artery from the from the right lower lobe, are isolated for study of vascular responsiveness. Cylindrical segments of each vessel are suspended between stainless steel hooks in 10-ml isolated tissue baths containing modified Krebs-Henseleit buffer aerated with 95% O_2/5% CO_2 at 37 °C. At the end of each experiment, vessel segments are blotted and weighed and their dimensions measured. Cross-sectional area of each artery is determined from tissue weight and diameter.

Arteries are equilibrated for 1 h at 1 g of passive applied load and then are made to contract to KCl (6×10^{-2} M). After washout, the procedure is repeated with applied loads increased by 1 g increments. Responses are normalized to the maximum active force development generated by an artery in each experiment and the data are plotted as a function of applied force. Changes in isometric force are monitored through force displacement transducers (Grass FT03) and recorded on a polygraph.

Responsiveness to contractile and relaxant agonists is assessed in pulmonary arteries from saline- and monocrotaline-treated rats both in verum- and placebo-treated groups. Cumulative concentration-response curves to hypertonic KCl, angiotensin II and norepinephrine are generated sequentially in vessels at resting tone. Arteries are then contracted submaximally with norepinephrine and cumulative concentration-response curves to the vasorelaxants isoproterenol and acetylcholine are determined.

EVALUATION

Contractions are expressed as active tension development, force generated per cross-sectional area and relaxations are normalized to precontraction tone. Both contractile and relaxation responses are plotted as a function of the negative logarithm of agonist concentration. Differences in mean responses are compared by a *t*-test for grouped data.

MODIFICATIONS OF THE METHOD

Molteni et al. (1986) treated rats continuously with monocrotaline in the drinking water at a concentration of 2.4 mg/kg/day for a period of 6 weeks. Test rats received an ACE-inhibitor during this time in the drinking water and controls the vehicle only. At the end of the experiment, hearts and lungs were weighed and examined by light and electron microscopy.

Madden et al. (1995) determined L-arginine-related responses to pressure and vasoactive agents in monocrotaline-treated rat pulmonary arteries.

Ono et al. (1995) studied the effects of prostaglandin E_1 (PGE_1) on pulmonary hypertension and lung vascular remodelling in the rat monocrotaline model of human pulmonary hypertension.

Yamauchi et al. (1996) studied the effects of an orally active endothelin antagonist on monocrotaline-induced pulmonary hypertension in rats.

Gout et al. (1999) evaluated the effects of adrenomedullin in isolated vascular rings from rats treated with monocrotaline (60 mg/kg s.c.) causing pulmonary hypertension and ventricular hypertrophy within 3 to 4 weeks.

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A.3
Coronary (cardiac) drugs

A.3.1
Isolated organs

A.3.1.1
Heart-lung preparation

PURPOSE AND RATIONALE

The isolated heart-lung of the dog was introduced by Knowlton and Starling (1912). Since then, the dog model has been used for many physiological and pharmacological studies (Krayer 1931; Krayer and Mendez 1942; Somani and Blum 1966; Takeda et al. 1973; Ishikawa et al. 1978, 1983; Ono et al. 1984a,b; Caffrey et al. 1986; Hausknecht et al. 1986; Fessler et al. 1988; Seifen et al. 1987, 1988; Naka et al. 1989). More recently, the rat model has been preferred (Dietz 1984, 1987; Onwochei et al. 1987, 1988; Kashimoto et al. 1987, 1990, 1994,1995, Fukuse et al. 1995).

PROCEDURE

Wistar rats weighing 300–320 g are anesthetized with 50 mg/kg pentobarbitone i.p. Tracheotomy is per-

formed and intermittent positive pressure ventilation is instituted with air. The chest is opened and flooded with ice-cold saline and the heart arrested. Cannulae are inserted into the aorta and the superior (for measurement of central venous pressure) and inferior venae cavae. The heart-lung preparation is perfused with a solution containing rat blood cells from another rat and Krebs-Ringer bicarbonate buffer, with hematocrit and pH of 25% and 7.4, respectively. Concentration of the buffer constituents (mM): NaCl 127, KCl 5.1, $CaCl_2$ 2.2, KH_2PO_4 1.3, $MgSO_4$ 2.6, $NaHCO_3$ 15, glucose 5.5 and heparin. The perfusate pumped from the aorta passes through a pneumatic resistance and is collected in a reservoir maintained at 37 °C and then returned to the inferior vena cava. In this model, no other organs except the heart and lung are perfused. Cardiac output is determined by the inflow as long as the heart does not fail. Mean arterial pressure is regulated by the pneumatic resistance. Heart rate is recorded by a bioelectric amplifier and cardiac output is measured with an electromagnetic blood flow meter. Arterial pressure and right atrial pressure are measured with transducers and amplifiers. The heart is perfused initially with cardiac output of 30 ml/min and mean arterial pressure of 80 mm Hg. Test drugs are administered into the perfusate 5 min after start of the experiment.

EVALUATION

Hemodynamic data within groups are analyzed by two-way analysis of variance (ANOVA) with repeated measures. Recovery time is measured by the Kruskal-Wallis test. The other data are analyzed by one-way ANOVA followed by the Dunnett test for multiple comparisons.

MODIFICATIONS OF THE METHOD

Using the Starling heart-lung preparation in dogs, Wollenberger (1947) studied the energy-rich phosphate supply of the failing heart.

Shigei and Hashimoto (1960) studied the mechanism of the heart failure induced by pentobarbital, quinine, fluoroacetate and dinitrophenol in dog's heart-lung preparation and effects of sympathomimetic amines and ouabain on it.

Imai et al. (1961) used heart-lung preparations of the dog to study the cardiac actions of methoxamine with special reference to its antagonistic action to epinephrine.

Capri and Oliverio (1965), Beaconsfield et al. (1974) used the heart-lung preparation of the **guinea pig**.

Robicsek et al. (1985) studied the metabolism and function of an autoperfused heart-lung preparation of the **dog**.

The **dog heart-lung preparation** was used by Seifen et al. (1988) to study the interaction of a calcium channel agonist with the effects of digoxin, by Somani and Blum (1966) to study blockade of epinephrine- and ouabain-induced cardiac arrhythmias in the dog,

by Riveron et al. (1988) to investigate the energy expenditure of an autoperfusing heart-lung preparation,

by Namakura et al. (1987) to study the role of pulmonary innervation in an *in situ* lung-perfusion preparation as a new model of neurogenic pulmonary edema,

by Hausknecht et al. (1986) to investigate the effects of lung inflation on blood flow during cardiopulmonary resuscitation,

by Caffrey et al. (1986) to evaluate the effect of naloxone on myocardial responses to isoproterenol,

by Ono et al. (1984) to estimate the cardiodepressant potency of various beta-blocking agents,

by Ishikawa et al. (1983) for a graphical analysis of drug effects in the dog heart-lung preparation – with particular reference to the pulmonary circulation and effects of norepinephrine and 5-hydroxytryptamine,

by Iizuka (1983) to study the cardiac effects of acetylcholine and its congeners,

by Fessler et al. (988) to investigate the mechanism of reduced LV afterload by systolic and diastolic positive pleural pressure,

by Takeda et al. (1973) to study the cardiac actions of oxprenolol.

Beaconsfield et al. (1974) used the heart-lung preparation of **guinea pigs** to study the cardiac effect of delta-9-tetrahydrocannabinol.

The **rabbit** autoperfusing heart-lung preparation was used by Muskett et al. (1986, 1988).

The isolated heart-lung preparation in the **cat** was described by Beaufort et al. (1993).

Kontos et al. (1987, 1988) harvested heart-lung blocks from **calves**.

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A.3.1.2 Isolated heart according to LANGENDORFF

PURPOSE AND RATIONALE

More than 100 years ago Langendorff (1895) described studies on isolated surviving mammalian hearts using mainly cats as donors. Since then, the method has been improved from the technical site and is nowadays used for studies with guinea-pig, rabbit or rat hearts. In principle, the heart is perfused in retrograde direction from the aorta either at constant pressure or at constant flow with oxygenated saline solutions. Retrograde perfusion closes the aortic valves, just as in the *in situ* heart during diastole. The perfusate is displaced through the coronary arteries flowing off the coronary sinus and the opened right atrium. In this original set-up the ventricles do not fill with perfusate and therefore do not perform pressure-volume work. Parameters usually measured are: contractile force, coronary flow and cardiac rhythm.

PROCEDURE

Guinea pigs of either sex weighing 300–500 g are sacrificed by stunning. For studies of biochemical parameters in tissue and perfusate, removal of the heart during barbiturate anesthesia and artificial respiration is recommended. The heart is removed as quickly as possible and placed in a dish containing Ringer's solution at 37 °C. Associated pericardial and lung tissue are removed. The aorta is located and cut just below the point of its division. A glass or plastic cannula is introduced into the aorta, tied with two threads and perfusion is started with oxygenated Ringer's solution or Krebs-Henseleit buffer. The heart is transferred to a double walled plexi-glass perfusion apparatus which is kept at 37 °C by the water from a thermostat. Oxygenated Ringer's solution is perfused at a constant pressure of 40 mm Hg and at a temperature of 37 °C from a reservoir. A small steel hook with a string is attached to the apex of the heart. Contractile force is measured isometrically by a force transducer with a preload of 2.5 g and recorded on a polygraph. Coronary flow is measured by a drop counter. Alternatively, flow measurements can be performed using a mechanic-electronic flow meter consisting of a vertical pipe and a magnetic valve (Hugo Sachs Electronic KG, Germany). Heart rate is measured through a chronometer coupled to the polygraph. Drugs are injected into the perfusion medium just above the aortic cannula.

CRITICAL ASSESSMENT OF THE METHOD

A reappraisal of the LANGENDORFF heart preparation was given by Broadley (1979) underlining the usefulness to test coronary vasodilating drugs. The value of the LANGENDORFF method can be best assessed by demonstrating a few of its applications in physiology and pharmacology. Direct effects can be measured as well as the antagonism against various physiological and pharmacological agents.

MODIFICATIONS OF THE METHOD

A survey on various modifications of the LANGENDORFF-technique and the **isolated working heart preparation** has been given by Ross (1972).

Neely et al. (1967) inserted a second cannula into a pulmonary vein or the left atrium. Perfusate from a reservoir flows via this cannula through the mitral valve into the left ventricle. During the systole of the heart, the left ventricle re-pumps the perfusate through the aorta into the reservoir. The perfusate flowing through the coronary arteries and dripping off from the outside of the heart is collected in a vessel below the heart and recirculated into the reservoir with a roller pump.

Flynn et al. (1978) underlined the difference of this working heart preparation to the original LANGENDORFF method and reported the effects of histamine and noradrenaline on peak left ventricular systolic pressure, contractility, sinus rate, coronary flow, aortic flow, total cardiac output, and external pressure-volume work. Therefore, this method is reported separately.

Ishiu et al. (1996) measured simultaneously Ca^{2+}-dependent indo-1 fluorescence and left ventricular pressure on a beat-to-beat basis in Langendorff guinea pig hearts and investigated the changes in Ca^{+2} transient and left ventricular function during positive inotropic stimulation and myocardial ischemia.

Hukovic and Muscholl (1962) described the preparation of the isolated **rabbit** heart with intact sympathic nervous supply from the right stellate ganglion.

Hendrikx et al. (1994) used the isolated perfused rabbit heart to test the effects of an Na^+/H^+ exchange inhibitor on postischemic function, resynthesis of high-energy phosphate and reduction of Ca^{2+} overload.

Michio et al. (1985) modified the Langendorff method in rabbits to a working heart preparation by cannulating the left atrium. At a pressure of 20 cm H_2O in the left atrium, the heart pumped the solution against a hydrostatic pressure of 100 cm H_2O. Aortic flow, systolic aortic pressure, coronary flow, and heart rate were measured.

The influence of an ACE-inhibitor on heart rate, lactate in the coronary effluate and GTP-level in the myocardium after 60-min hypothermic cardiac arrest was studied in working heart preparation of rabbits by Zegner et al. (1996).

Gottlieb and Magnus (1904) introduced the so called **"balloon method"**. A small balloon fixed to the tip of

a catheter is filled with water and inserted into the left ventricle via one of the pulmonary veins, the left atrium and the mitral valve. The balloon size has to fit the volume of the left ventricle and therefore its size depends on the animal species and body weight. The catheter can be fixed by tying the pulmonary vein stems. Via a three-way valve, the balloon can be extended to a given preload. The beating heart now exerts a rhythmic force to the balloon and thus to the membrane of a pressure transducer. The advantages of this method are that force development and preload can be stated reproducibly in pressure units [mm Hg], left ventricular contraction curves can be used for further calculations, and continuous heart rate recordings can be carried out without any problems when using a rate meter.

Sakai et al. (1983) reported a similar method adapted to **mice**.

Bardenheuer and Schrader (1983) described a method whereby the balloon is inserted into the left ventricle as described above. However, isovolumetric pressure in the left ventricle is not measured. Instead, the fluid in the balloon is pumped through the cannula into a closed extra corporal circulation. The fluid is forced into one direction by 2 recoil valves. The balloon is made of silicone material using a Teflon form (Linz et al. 1986). The dimensions of the form are derived from casts of the left ventricle of K^+-arrested heart by injection of dental cement (Palavit 55®, Kulzer and Co, GmbH, Germany). During each heart beat the fluid volume expelled from the balloon corresponding to the stroke volume of the heart, can be recorded by means of a flow meter probe and an integrator connected in series. Preload and afterload can be adjusted independently from each other. The perfusate flow (retrograde into the aorta and through the coronary arteries) is recorded separately.

The following parameters were measured in isolated **rat** hearts (Linz et al. 1986; Linz et al. 1990):

- LVP (left ventricular pressure) with Statham pressure transducer P 23 DB, which on differentiation yielded LV dp/dt_{max} and HR (heart rate). Cardiac output and coronary flow (CF) are determined by electromagnetic flow probes in the outflow system and in the aortic cannula, respectively. Coronary venous pO_2 is measured with a catheter placed in the pulmonary artery by a type E 5046 electrode connected to a PMH 73 pH/blood gas monitor (Radiometer). An epicardial electrocardiogram recording is obtained via two silver electrodes attached to the heart. All parameters are recorded on a Brush 2600 recorder.
- Myocardial oxygen consumption (MWO_2) [ml/min/g wet weight] is calculated according to the equation:

$$MVO_2 = CF \times (P_a - P_v) \times (c/760) \times 100$$

where CF is the coronary flow [ml/min/g], P_a is the oxygen partial pressure of arterial perfusate (650 mm Hg), P_v is the oxygen partial pressure of the venous effluent perfusate [mm Hg], and c is the 0.0227 ml O_2/ml perfusate representing the Bunsen solubility coefficient of oxygen dissolved in perfusate at 37 °C (Zander and Euler 1976).

For the determination of lactate dehydrogenase (LDH) and creatine kinase (CK) activities in the perfusate, samples are taken from the coronary effluent.

After the experiments, hearts are rapidly frozen in liquid nitrogen and stored at –80 °C. Of the left ventricle, 500 mg are taken, put into 5 ml ice-cold $HClO_4$ and disrupted with an Ultra-Turrax (Junke and Kunkel, Ika-Werk, Type TP). Glycogen is hydrolyzed with amyloglycosidase (pH 4.8) and determined as glucose. Furthermore, ATP and creatine phosphate are measured.

Avkiran and Curtis (1991) constructed a dual lumen aortic cannula which permits independent perfusion of left and right coronary beds in isolated rat hearts without necessitating the cannulation of individual arteries.

Igic (1996) described a modification of the isolated perfused working rat heart. A special double cannula was designed consisting of an outer cannula that is inserted into the aorta and an inner cannula that is advanced into the left ventricle. The perfusion fluid flows through the inner cannula into the left ventricle, and is ejected from there into the aorta. If the outer cannula system is closed, the fluid perfuses the coronary vessels and drips off outside the heart. When the outer cannula is open and certain pressure resistance is applied, a fraction of the ejected fluid perfuses coronary vessels and the rest is expelled. Because the inner cannula can be easily retracted into the outer cannula, which is placed in the aorta, the preparation provides an opportunity to use the same heart as a "working" or "non-working" model for investigating functions of the heart.

By labeling glucose, lactate, or fatty acids in the perfusate with ^3H or ^{14}C, Barr and Lopaschuk (1997) directly measured energy metabolism in the isolated rat heart.

Krzeminski et al. (1991) described a new concept of the isolated heart preparation with on-line computerized data evaluation. Left ventricular pressure was recorded by means of a balloon-catheter, while special suction electrodes obtained the high-amplitude, noise-free electrogram recordings. The coronary effluent partial pressure of oxygen was continuously monitored, which enabled the calculation of myocar-

dial oxygen consumption (MVO_2). The effluent partial pressure of carbon dioxide and pH value were also measured simultaneously. A computerized system of data acquisition, calculation, storage, and end report was described.

Döring and Dehnert (1988) described continuous simultaneous **ultrasonic recording** of two cardiac diameters in an isolated perfused guinea-pig heart. For the measurement of the left ventricular transversal diameter the ultrasonic transmitter was positioned at the epicardium at the largest cardiac diameter. The corresponding ultrasonic receiver was inserted through the right atrium into the right ventricle to approximately the same height as the transmitter. In the right ventricle, which is empty in the isolated perfused LANGENDORFF-heart, it was automatically positioned opposite to the transmitter. Additional transducers were placed both at the heart's base and apex for assessment of the ventricular longitudinal diameter.

Several authors used the **isolated perfused mouse heart**.

Bittner et al. (1996) described a work-performing heart preparation for myocardial performance analysis in murine hearts using a modified Langendorff apparatus.

Sumaray and Yellon (1998a,b) constructed a specially designed Langendorff apparatus that allows perfusion of the isolated **mouse** heart. These authors reported that ischemic preconditioning reduces infarct size following global ischemia in the murine myocardium.

Brooks and Apstein (1996) measured left ventricular systolic and diastolic pressures in the isovolumically contracting (balloon in the left ventricle) mouse hearts.

Plumier et al. (1995) generated **transgenic mice** expressing the human heart heat shock protein 70. Upon reperfusion of the hearts after 30 min of ischemia in the Langendorff preparation, transgenic hearts versus non transgenic hearts showed significantly improved recovery of contractile force.

Hannan et al. (2000) compared ENOS knockout and wild-type mouse hearts which were perfused in a Langendorff apparatus with Krebs bicarbonate buffer and subjected to 20 min of global normothermic ischemia followed by 30 min of reperfusion. Myocardial function was measured using a ventricular balloon to determine time to onset of contraction, left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), and heart rate-pressure product (RPP).

Sheikh et al. (2001) generated transgenic mice overexpressing fibroblast growth factor (FGF)-2 protein in the heart. An isolated mouse heart model of ischemia-reperfusion injury was used to assess the potential of endogenous FGF-2 for cardioprotection.

APPLICATIONS

Positive inotropic effects

While negative inotropic substances can be tested in a heart beating with normal force, the evaluation of a positive inotropic compound usually requires that cardiac force is first reduced. Acute experimental heart failure can be induced by an overdose of barbiturates, such as sodium thiopental, or calcium antagonists. This kind of cardiac failure can be reversed by β-sympathomimetic drugs, cardiac glycosides, or increased Ca^{+2} concentration. In this way, the potential β-sympathomimetic activity of a new drug can be measured using isoproterenol as standard. After thiopental-Na treatment, left ventricular pressure (LVP) and dp/dt_{max} decrease considerably, whereas coronary flow is slightly enhanced. β-Sympathomimetic drugs restore LVP and dp/dt_{max} and keep coronary blood flow elevated.

Cardiac glycosides increase LVP and dp/dt_{max} and leave coronary flow unchanged.

Negative inotropic effects

The effects of a β-sympathomimetic drug such as isoproterenol at doses of 0.05 to 0.2 µg increasing contractile force as well as heart frequency are registered. After injection of a β-blocker, the effects of isoproterenol are attenuated. The effects of a potential β-blocking agent can be tested comparing the isoproterenol inhibition versus a standard such as propranolol (0.1 mg).

Coronary vessel dilating effect

The LANGENDORFF heart has been extensively used for assessing the coronary dilating activity of drugs (Broadley 1979). Rothaul and Broadley (1982) demonstrated the release of coronary vasodilator mediators from guinea pig isolated hearts by a technique employing donor and recipient hearts in series.

Calcium-antagonism

In order to demonstrate the effect of calcium-antagonists, 1 to 5 mg $BaCl_2$ are injected which induce a pronounced spasm of the coronary arteries thereby reducing the coronary flow. Five min later, the test drug is injected. Active compounds have a relaxing effect on coronary arteries indicated by an increase of coronary flow. After this effect has weaned, $BaCl_2$ is injected again and the test drug or a standard drug, e.g. nifedipine, is tested. The increase of coronary flow is expressed as percentage of flow during $BaCl_2$ spasm and compared with the effect of the standard. Using various doses, dose-response curves can be established.

Effect on potassium outflow induced by cardiac glycosides

Lindner and Hajdu (1968) described a method using the LANGENDORFF heart in which contractile force, coronary flow, and the potassium content in the coronary outflow was determined by flame photometry. Increase in potassium outflow correlates well with the positive inotropic effect.

Gradual determination of hypoxic damage

Lindner and Grötsch (1973) measured the enzymes creatine phosphokinase (CPK), lactate dehydrogenase (LDH), α-hydroxy-butyrate dehydrogenase (α-HBDH), and glutamic-oxalacetic transaminase (GOT) in the effluent of a guinea pig heart preparation under varying degrees of hypoxia. Potassium content and oxygen tension in the inflowing and outflowing solution were determined. The heart rate, the amplitude of contraction and the rate of coronary vessel perfusion were recorded additionally.

Metabolic studies with nuclear magnetic resonance

Using ^{31}P, studies on metabolism of nucleotides and phosphorylated intermediates of carbohydrates in isolated hearts have been performed (Garlick et al. 1977; Jacobus et al. 1977; Hollis et al. 1978; Matthews and Radda 1984).

Arrhythmogenic, anti-arrhythmic and antifibrillatory effects

The LANGENDORFF heart preparation is also used to test the influence of compounds on cardiac rhythm. For recording monophasic action potentials, suction electrodes are applied on the heart. Ventricular fibrillation can be induced by simultaneous injection of digitoxin (12.5–25.0 µg) and aconitine (12.5–25.0 µg) into the perfusion fluid (Lindner 1963). Cardiac glycosides shorten the refractory period, decrease the conduction velocity and increase heterotopic stimulus generation. Aconitine increases markedly heterotopic stimulus generation. Both compounds together induce invariably ventricular fibrillation. Anti-arrhythmic compounds can be tested in this way. Fibrillation is inhibited, at least partially, by 20 µg prenylamine, 10–20 µg quinidine or 20 µg ajmaline.

Takeo et al. (1992) described protective effects of anti-arrhythmic agents on oxygen-deficiency-induced contractile dysfunction of isolated perfused hearts. Hypoxia in isolated rabbit hearts was induced by perfusing the heart for 20 min with Krebs-Henseleit buffer saturated with a gas mixture of 95% N_2 and 5% CO_2 containing 11 mM mannitol. After hypoxic perfusion, the heart was reoxygenated for 45 min with oxygenated buffer containing glucose.

Dhein et al. (1989) studied the pathway and time course of the epicardial electrical activation process by means of a computer-assisted epicardial potential mapping, using a matrix of 256 unipolar AgCl electrodes (1 mm spatial and 0.25 ms temporal resolution) in isolated rabbit hearts perfused according to the Langendorff technique. From the activation times of the surrounding electrodes, the direction and velocity of activation for each electrode were calculated, thereby allowing construction of an epicardial vector field. The method was used for the assessment of arrhythmogenic and anti-arrhythmic drug activity.

Electrical stimulation and antifibrillatory effect

Ventricular fibrillation can be induced in the LANGENDORFF preparation by reducing the glucose content of the perfusion medium to 0.25 g/1 000 ml and the KCl content to 0.12 g/1 000 ml. (Burn et al. 1957, 1960; Lindner 1963). After a perfusion period of 20 min, 10 µg epinephrine are injected into the perfusion cannula. Immediately afterwards, the heart is stimulated with a current of 40 Hz and 5 mA for 2 min. This procedure is repeated every 10 min. Standard conditions are achieved when the fibrillation continues without further electrical stimulation. Hearts treated in this way serve as controls. Other hearts stimulated in the same way are treated with continuous infusion of the test drug or the standard via the perfusion medium. Differences in the incidence of fibrillations are calculated using the χ^2 test.

Electrophysiological evaluation of cardiovascular agents

Balderston et al. (1991) modified the Langendorff technique in rabbit hearts in order to perform electrophysiologic studies. His bundle electrograms were measured with a plunge electrode and allowed atrioventricular nodal physiology to be evaluated directly. Atrial conduction and refractoriness, atrioventricular node conduction and refractoriness, His-Purkinje conduction, and ventricular conduction and refractoriness could be accurately measured. The effects of verapamil and flecainide were described.

EDRF release from the coronary vascular bed

Lamontagne et al. (1992) isolated platelets from blood of healthy human donors and injected platelets boluses into the perfusion line of the Langendorff preparation of a rabbit heart. In the effluent cyclic GMP was determined as an index for EDRF release.

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A.3.1.3 Coronary artery ligation in isolated working rat heart

PURPOSE AND RATIONALE

In working heart preparations of rats, ischemia can be induced by clamping the left coronary artery close to its origin. After removal of the clip, changes in the reperfusion period can be observed. Prevention of these symptoms can be an indicator of the efficacy of coronary drugs.

PROCEDURE

The preparation used is a modification of an isolated working heart preparation originally used for guinea pig hearts (Bardenheuer and Schrader 1983). Wistar rats of either sex weighing 280–300 g are sacrificed by decapitation. The hearts are removed and dissected free from the epicard and surrounding connective tissue. A cannula is introduced into the aorta from where the coronary vessels are perfused with the non-recirculated perfusion medium according to the Langendorff technique. In the left ventricle a balloon closely fitting the ventricular cavity is placed and connected to an artificial systemic circulation. The fluid in the balloon is pumped through a cannula into the closed extra corporal circulation being forced into one direction by 2 recoil valves. The balloon is made of silicone material using a Teflon form (Linz et al. 1986). The dimensions of the form are derived from casts of the left ventricle of K^+-arrested heart by injection of dental cement (Pala-vit 55®, Kulzer and Co, GmbH, Germany). During each heart beat the fluid volume pressed from the balloon, corresponding to the stroke volume of the heart, can be recorded by means of a flow meter probe and an integrator connected in series. Preload and afterload can be adjusted independently from each other. The per-fusate flow (retrograde into the aorta and through the coronary arteries) is recorded separately.

The following parameters were measured in isolated rat hearts (Linz et al. 1986):

LVP (left ventricular pressure) with Statham pressure transducer P 23 DB, which on differentiation yielded LV dp/dt_{max} and HR (heart rate). Cardiac output and coronary flow (CF) are determined by electromagnetic flow probes in the outflow system and in the aortic cannula, respectively. Coronary venous pO_2 is measured with a catheter placed in the pulmonary artery by a type E 5046 electrode connected to a PMH 73 pH/blood gas monitor (Radiometer). An epicardial electrocardiogram recording is obtained via two silver electrodes attached to the heart. All parameters are recorded on a Brush 2600 recorder.

Myocardial oxygen consumption (MWO_2) [ml/min/g wet weight] is calculated according to the equation:

$$MVO_2 = CF \times (P_a - P_v) \times (c/760) \times 100$$

where CF is the coronary flow [ml/min/g], P_a is the oxygen partial pressure of arterial perfusate (650 mm Hg), P_v is the oxygen partial pressure of the venous effluent perfusate [mm Hg], and c is the 0.0227 ml O_2/ml perfusate representing the Bunsen solubility coefficient of oxygen dissolved in perfusate at 37 °C (Zander and Euler 1976).

Coronary artery ligation

For coronary artery occlusion experiment (Scholz et al. 1992, 1993), the isolated working hearts are perfused for a period of 20 min (pre-ischemic period) with modified Krebs-Henseleit buffer at a constant pressure of 65 mm Hg. Thereafter, acute myocardial ischemia is produced by clamping the left coronary artery close to is origin for 15 min (ischemic period). The clip is then reopened, and changes during reperfusion are moni-

tored for 30 min (reperfusion period). After coronary artery ligation and reperfusion the hearts develop ventricular fibrillation.

From the coronary effluent samples are taken for lactate, lactate dehydrogenase (LDH), and creatine kinase (CK) determinations. After the experiment, glycogen, lactate, ATP, and creatine phosphate in myocardial tissue are measured.

The test drugs are given into the perfusion medium either before occlusion or 5 min before reperfusion. For *ex vivo* studies, the rats are treated orally with the test drug 1 h before sacrifice and preparation of the isolated working heart.

EVALUATION

The incidence and duration of ventricular fibrillation after treatment with coronary drugs is compared with controls. Left ventricular pressure, LV *dP/dt* max, and coronary flow are reduced after coronary constriction by angiotensin II, whereas enzyme activities in the effluent are increased and the myocardial content of glycogen, ATP and creatine phosphate are decreased. Cardiac protective drugs have the opposite effects. The values of each parameter are statistically compared with controls.

MODIFICATIONS OF THE METHOD

Vogel and Lucchesi (1980) described an isolated, blood perfused, **feline** heart preparation for evaluating pharmacological interventions during myocardial ischemia. Ventricular function was measured with a fluid-filled latex balloon within the left ventricle.

Vleeming et al. (1989) ligated the left coronary artery in rats after thoracotomy in ether anesthesia. Forty-eight hours after the operation, the hearts were prepared for retrograde constant pressure perfusion, according to the Langendorff technique.

Igic (1996) presented a new method for the isolated working rat heart. A special double cannula was designed consisting of an outer cannula that is inserted in the aorta and an inner cannula that is advanced into the left ventricle. The perfusion fluid flows through the inner cannula into the left ventricle, and is ejected from there into the aorta. If the outer cannula system is closed, the fluid perfuses the coronary vessels and drips off outside the heart. When the outer cannula is open and certain pressure resistance is applied, a fraction of the ejected fluid perfuses the coronary vessels and the rest is expelled. Because the inner cannula can easily be retracted into the outer cannula, which is placed in the aorta, this preparation provides an opportunity to use the same heart as a "working" or "non-working" model for investigating functions of the heart.

Pepe and McLennan (1993) described a maintained afterload model of ischemia in erythrocyte-perfused isolated working hearts of rats.

Further characterization of the pathophysiological reactions or the isolated working heart was performed by Linz et al. (1999). The external heart power (EHP) [mJ/min/g] was calculated using the formula:

$$EHP_{LV} = \text{pressure} - \text{volume work} + \text{acceleration work}$$

$$= \{[SV\,(MAP - LAP)] + [1/2\,SV \times d \times (SV/\pi\, r^2\, e^2)]\}\, HR\, g_{LVwwt}{}^{-1}$$

SV indicates stroke volume; *MAP*, mean aortic pressure; *LAP*, mean left arterial pressure; *d*, specific weight perfusate (1.004 g/cm^3); *r*, inner radius of aortic cannula; *e*, ejection time; *HR*, heart rate; LV, left ventricle; LVwwt, left ventricular wet weight.

The function of the left ventricle was altered by changing the aortic pressure (afterload) at constant left atrial filling load (preload). By adjusting the Starling resistance, the aortic outflow could be switched during 1 min from the fixed baseline afterload to a preset higher afterload producing step-wise rises in mean arterial pressure.

Lee et al. (1988) studied the effects of acute global ischemia on cytosolic calcium transients in perfused isolated **rabbit** hearts with the fluorescent calcium indicator indo 1. Indo 1-loaded hearts were illuminated at 360 nm, and fluorescence was recorded simultaneously at 400 and 550 nm from the epicardial surface of the left ventricle. The F_{400}/F_{550} ratio was calculated by an analog circuit, which allowed cancellation of optical motion artifact. The resulting calcium transients were registered simultaneously with the ventricular pressure and demonstrated a rapid upstroke and slow decay similar to those recorded in isolated ventricular myocytes. Global ischemia rapidly suppressed contraction, but it produced a concurrent increase in the systolic and diastolic levels of calcium transients, together with an increase in the duration of the peak.

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A.3.1.4
Relaxation of bovine coronary artery

PURPOSE AND RATIONALE

Eicosanoids can regulate the tonus of coronary arteries. Prostacyclin induces relaxation, whereas thromboxane A_2 causes contraction. Spiral strips from bovine coronary artery can be used for assaying relaxation activity of test compounds (Dusting et al. 1977)

PROCEDURE

Freshly slaughtered beef hearts are immersed in cold oxygenated Krebs solution and immediately transported in a thermos flask to the laboratory. The left descending coronary artery and several of its primary branches are cut into spiral strips (about 20 mm long and 2–3 mm wide). The specimens can be stored up to 48 h at 4 °C. The artery strips are suspended in a 4 ml organ bath under an initial tension of 2 g and immersed in a Krebs' bicarbonate solution at 37 °C being gassed with oxygen containing 5% CO_2 throughout the experiment. The Krebs solution contains a mixture of antagonists to inhibit any actions from endogenous acetylcholine, 5-hydroxytryptamine, histamine or catecholamines (hyoscine hydro-bromide 10^{-7} g/ml, methysergide maleate 2×10^{-7} g/ml, mepyramine maleate 10^{-7} g/ml, propranolol hydrochloride 2×10^{-6} g/ml). The strips are superfused with a solution of the test compounds in concentrations of 0.01, 0.1, 1.0 µg/ml at a rate of 10–20 ml/min with oxygenated Krebs solution containing the mixture of antagonists. Isometric contractions are recorded with Grass force-displacement transducers (type FT 03 C) on a Grass polygraph. The strips are superfused with Krebs' solution 3 h prior to the experiment. Standard compounds are 100 ng/ml PGE_2 inducing contraction and 100 ng/ml PGI_2 inducing pronounced relaxation.

EVALUATION

The relaxation induced by the test compound is expressed as percentage of maximal response to 100 ng/ml PGI_2.

MODIFICATIONS OF THE METHOD

Campell and Paul (1993) measured the effects of diltiazem on isometric force generation, $[Ca^{2+}]_i$, and energy metabolism in the isolated **porcine** coronary artery.

Li et al. (1997) determined the ability of analogues of human α-calcitonin gene-related peptide to relax isolated porcine coronary arteries precontracted with 20 mM KCl.

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A.3.2 *In vivo* methods

A.3.2.1 Isoproterenol induced myocardial necrosis in rats

PURPOSE AND RATIONALE

Cardiac necrosis can be produced by injection of natural and synthetic sympathomimetics in high doses. Infarct-like myocardial lesions in the rat by isoproterenol have been described by Rona et al. (1959). These lesions can be totally or partially prevented by several drugs such as sympatholytics or calcium-antagonists.

PROCEDURE

Groups of 10 male Wistar rats weighing 150–200 g are pretreated with the test drug or the standard either s.c. or orally for 1 week. Then, they receive 5.25 and 8.5 mg/kg isoproterenol s.c. on two consecutive days. Symptoms and mortality in each group are recorded and compared with those of rats given isoproterenol alone. Forty-eight hours after the first isoproterenol administration, the rats are sacrificed and autopsied. The hearts are removed and weighed, and frontal sections are embedded for histological examination.

EVALUATION

Microscopic examination allows the following grading:

Grade 0: no change
Grade 1: focal interstitial response
Grade 2: focal lesions in many sections, consisting of mottled staining and fragmentation of muscle fibres
Grade 3: confluent retrogressive lesions with hyaline necrosis and fragmentation of muscle fibres and sequestrating mucoid edema
Grade 4: massive infarct with occasionally acute aneurysm and mural thrombi

For each group the main grade is calculated with the standard deviation to reveal significant differences.

CRITICAL ASSESSMENT OF THE METHOD

The test has been used by many authors for evaluation of coronary active drugs, such as calcium-antagonists and other cardioprotective drugs like nitroglycerin and molsidomine (Vértesy et al. 1991; Classen et al. 1993).

MODIFICATIONS OF THE METHOD

Yang et al. (1996) reported a protective effect of human adrenomedullin[13–52], a C-terminal fragment of adrenomedullin[10–52] on the myocardial injury produced by subcutaneous injection of isoproterenol into rats.

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A.3.2.2 Myocardial infarction after coronary ligation

PURPOSE AND RATIONALE

Ligation of the left coronary artery in rats as described by Selye (1960) induces an acute reduction in pump function and a dilatation of left ventricular chamber. The method has been used to evaluate beneficial effects of drugs after acute (Chiariello et al. 1980; Flaim and Zelis 1981; Bernauer 1985) or chronic (Innes and Weisman 1981; Pfeffer et al. 1985; Linz et al. 1996) treatment.

PROCEDURE

Male Sprague Dawley rats weighing 200–300 g are anesthetized with diethyl-ether. The chest is opened by a left thoracotomy, and a thread is inserted near the middle of the lateral margin of the cutaneous wound and carried through a tunnel of the left pectoral muscle around the cranial half of the incision. The heart is gently exteriorized by pressure on the abdomen. A ligature is placed around the left coronary artery, near its origin, and is tightened. Within seconds, the heart is repositioned in the thoracic cavity, and the ends of the musculocutaneous thread are tightened to close the chest wall and enable the animal to breathe spontaneously. The speed of the procedure renders mechanical respiration unnecessary.

To evaluate drug effects, the rats are treated 5 min after and 24 h after occlusion by subcutaneous injection (standard 5 mg/kg propranolol).

Two days after surgery, the rats are anesthetized with 60 mg/kg i.p. pentobarbital and the right carotid artery is cannulated with a polyethylene catheter connected to a pressure transducer. The fluid-filled catheter is then advanced into the left ventricle through the aortic valve for measurement of left ventricular systolic and end-diastolic pressure.

After hemodynamic measurements, the heart is arrested by injecting 2 ml of 2.5 M potassium chloride. The chest is opened, and the hearts are isolated and rinsed with 300 mM KCl to maintain a complete diastole. A double-lumen catheter is advanced into the left ventricle through the ascending aorta, the right and left atria are tied off with a ligature, and the right ventricle is opened. The left ventricular chamber is filled with a cryostatic freeze medium through the smaller of the two catheter lumens and connected to a hydrostatic pressure reservoir maintained at a level corresponding to the end-diastolic pressure measured *in vivo*. The outlet (larger lumen) is then raised to the same level as the inlet to allow fluid in the two lumens to equilibrate. The heart is rapidly frozen with hexane and dry-ice.

The hearts are serially cut with a cryostat into 40-µm-thick transverse sections perpendicularly to the longitudinal axis from apex to base. At a fixed distance, eight sections are obtained from each heart and collected on gelatin-coated glass slides. Sections are air-dried and incubated at 25 °C for 30 min with 490 µM nitroblue tetrazolium and 50 mM succinic acid in 0.2 M phosphate buffer (pH 7.6), rinsed in cold distilled water, dehydrated in 95% ethyl alcohol, cleared in xylene, and mounted with a synthetic resin medium. Viable tissue appears dark blue, contrasting with the unstained necrotic tissue.

EVALUATION

The infarct size can be determined by planimetry and expressed as percentage of left ventricular area, and thickness can be expressed as percentage of non-infarcted ventricular wall thickness (MacLean et al. 1978; Chiariello 1980; Roberts et al. 1983). An automatic method for morphometric analysis with image acquisition and computer processing was described by Porzio et al. (1995).

CRITICAL ASSESSMENT OF THE METHOD

Hearse et al. (1988) challenged the value of the model of coronary ligation in the rat because of inappropriate interpretations.

MODIFICATIONS OF THE METHOD

Johns and Olson (1954) described the coronary artery patterns for mouse, rat, hamster and guinea pig.

Kaufman et al. (1959), Fishbein et al. (1978, 1980) used various histochemical methods for identification and quantification of border zones during the evolution of myocardial infarction.

Ytrehus et al. (1994) analyzed the effects of anesthesia, perfusate, risk zone, and method of infarct sizing in rat and rabbit heart infarction.

Scholz et al. (1995) described a dose-dependent reduction of myocardial infarct size in rabbits by a selective sodium-hydrogen exchange subtype 1 inhibitor.

Chiariello et al. (1976) compared the effects of nitroprusside and nitroglycerin on ischemic injury during acute myocardial infarction in dogs.

Leprán et al. (1981) placed a loose ligature of atraumatic silk around the left anterior descending coronary artery under ether anesthesia in rats. Ten days later, acute myocardial infarction was produced by tightening the ligature.

Gomoll and Lekich (1990) discussed the use of the ferret for a myocardial ischemia/salvage model.

Holmborn et al. (1993) compared triphenyltetrazolium chloride staining versus detection of fibronectin in experimental myocardial infarction in pigs.

Michael et al. (1995) described in detail the surgical procedure to induce myocardial ischemia in mice by ligation of the left anterior descending branch of the left coronary artery.

Kouchi et al. (2000) found an increase in $G_{i\alpha}$ protein accompanying progression of post-infarction remodelling in hypertensive cardiomyopathy in rats. G protein α subunits were studied with immuno-blotting techniques (Böhm et al. 1990). The polyclonal antiserum MB1 was raised in rabbits against the carboxyl-terminal decapeptide of retinal transduction (KENLKDCGLF) coupled to keyhole limpet hemocyanine. The MB1 recognized $G_{i\alpha1}$ and $G_{i\alpha2}$ but not $G_{0\alpha}$ and $G_{i\alpha3}$ (Böhm et al. 1994). The membrane fractions were electrophoresed in SDS-polyacrylamide gels and were transferred to nitrocellulose filters. The filters were incubated with the first antibodies for $G_{i\alpha}$ (MB1) or $G_{s\alpha}$ (RM/1) and then with the second antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Amersham). Immunoreactive signals were detected by means of the ECL kit (Amersham).

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A.3.2.3 Occlusion of coronary artery in anesthetized dogs

PURPOSE AND RATIONALE

The size of infarcts is studied after proximal occlusion of the left anterior descending coronary artery in open-chest dogs. Compounds potentially reducing infarct-size are tested. To delineate the post-mortem area at risk, coronary arteriograms are made after injection of a $BaSO_4$-gelatin mass into the left coronary ostium. The infarct's area is visualized with nitro-blue tetrazolium chloride in myocardial sections.

PROCEDURE

Dogs of either sex weighing approximately 30 kg are used. The animals are anesthetized by intravenous injection of pentobarbital sodium (bolus of 35 mg/kg followed by continuous infusion of 4 mg/kg/h). The animals are placed in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator. Arterial blood gases are checked, and the ventilation rate and/or oxygen flow rate are adjusted to achieve physiological blood gas values (P_{O2}: 100–140 mm Hg, P_{CO2}: 32–40 mm Hg, and pH 7.47). A peripheral vein (saphenous vein) is cannulated for the administration of test compound. The ECG is recorded continuously from lead II (Einthoven).

Preparation for hemodynamic measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of a femoral vein is connected to a pressure transducer (Statham P 23 DB). For determination of left ventricular pressure (LVP), a Millar microtip catheter (PC 350) is inserted via the left carotid artery. Left ventricular enddiastolic pressure (LVEDP) is measured from a high-sensitivity scale. From the pressure curve, *dp*/*dt* max is differentiated and heart rate is counted.

Experimental procedure

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal space, the pericard is opened and the left anterior descending coronary artery (LAD) is exposed. After reaching steady state conditions for the hemodynamic parameters (approx. 45 min), the LAD is ligated just below the first diagonal branch for 360 min. No attempt is made to suppress arrhythmic activity after the ligation.

The test substance or the vehicle (controls) is administered by intravenous bolus injection and/or continuous infusion. The schedule of administration may vary. Hemodynamic parameters are registered continuously during the whole experiment. At the end of the experiment, the animals are sacrificed with an overdose of pentobarbital sodium and the heart is dissected.

Preparation to determine area at risk

Coronary arteriograms are made according to Schaper et al. (1979) to delineate the anatomic post-mortem area at risk. A purse-string suture is placed around the left coronary ostium in the sinus of Valsalva; a cannula is then placed in the ostium and the purse-string suture is tightened. Micronized $BaSO_4$ suspended in 12% gelatin solution (37 °C) is injected under increasing pressure (2 min at 100 mm Hg, 2 min at 150 mm Hg and 2 min at 200 mm Hg). The heart is placed in crushed ice to gel the injectate. The right ventricle is removed and the left ventricle plus septum is cut into transverse sections (approx. 1 cm thick) from the apex to the level of the occlusion (near the base). From each slice angiograms are made with a X-ray tube at 40 kV to assess the post-mortem area at risk (by defect opacity: reduction of $BaSO_4$-filled vessels in infarct tissue).

Preparation to determine infarct size

The slices are then incubated in p-nitro-blue tetrazolium solution (0.25 g/L in Sörensen phosphate buffer, pH 7.4, containing 100 mM D,L-maleate) in order to visualize the infarct tissue (blue/violet-stained healthy tissue, unstained necrotic tissue). The slices are photographed on color transparency film for the determination of the infarct area.

Left ventricle and infarct area, and area at risk are measured by planimetry from projections of all slices with the exclusion of the apex and of the slice containing the ligature.

EVALUATION

Mortality and the different hemodynamic parameters are determined. Changes of parameters in drug-treated animals are compared to vehicle controls. The different characteristics are evaluated separately. Mean values ±SEM of infarct area and of area at risk are calculated. Statistical analyses consist of regression and correlation analyses and of the Student's *t*-test. Results are considered significant at $p < 0.05$.

MODIFICATIONS OF THE METHOD

Nachlas and Shnitka (1963) described the macroscopic identification of early myocardial infarcts by alterations in dehydrogenase activity in dogs by staining the cardiac tissue with Nitro-BT [2,2'-di-p-nitrophenyl-5,5'diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride] yielding a dark blue formazan in viable muscle but not in necrotic muscle fibers.

Black et al. (1995) studied the cardioprotective effects of heparin or N-acetylheparin in an *in vivo* dog model of myocardial and ischemic reperfusion injury.

The hearts were removed after 90 min of coronary occlusion and a 6 h-reperfusion period. Area at risk was determined by the absence of Evans blue dye after perfusion of the aorta in a retrograde fashion and infarct zone by the absence of formazan pigment within the area at risk after perfusion of the circumflex coronary artery with triphenyltetrazolium chloride.

Reimer et al. (1985) tested the effect of drugs to protect ischemic myocardium in unconscious and conscious dogs. In the conscious model, dogs of either sex weighing 10–25 kg were anesthetized with thiamyal sodium (30–40 mg/kg i.v.) and underwent thoracotomy through the 4th intercostal space. Heparin-filled polyvinyl chloride catheters were positioned in the aortic root, the left atrium via the left atrial appendage, and a systemic vein. A mechanical adjustable snare type occluder was placed around the proximal left circumflex coronary artery above or below the first marginal branch, so that temporary occlusion resulted in cyanosis of at least 75% of the inferior wall. The catheters and snare were either exteriorized or positioned in a subcutaneous pocket at the back of the neck. Penicillin, 1 000 000 units, and streptomycin, 1.0 g, were given i.m. for the first 4 postoperative days, and at least 7 days were allowed for recovery from surgery.

Dogs were fasted overnight prior to the study. After exteriorization and flushing of the catheters, 30–40 min were allowed for the animals to adjust to laboratory conditions. Morphine sulfate, 0.25 mg/kg, i.m., was given 30 min before occlusion, and an additional 0.25 mg/kg, i.v., was given 20 min later. Heart rate and aortic and left atrial pressures were monitored continuously. Permanent coronary occlusion was produced by a sudden one-stage tightening of the snare occluder. Drugs were administered by continuous i.v. infusion over 6 h. Hemodynamic measurements were taken 5 min before occlusion and 10, 25, 105, 180, and 360 min after occlusion.

Raberger et al. (1986) described a model of **transient myocardial dysfunction in conscious dogs**. Mongrel dogs, trained to run on a treadmill, were chronically instrumented with a miniature pressure transducer in the left ventricle and a hydraulic occluder placed around the circumflex branch of the left coronary artery. Two pairs of piecoelectrical crystals for sonomicrometry were implanted subendocardially to measure regional myocardial functions. Comparable episodes of regional dysfunction of the left coronary artery area during treadmill runs were found after partial left coronary artery stenosis induced by external filling of the occluder.

Hartman and Warltier (1990) described a model of **multivessel coronary artery disease** using conscious, chronically instrumented dogs. A hydraulic occluder was implanted around the left anterior descending coronary artery (LAD) and an Ameroid constrictor around the left circumflex coronary artery (LCCA). Pairs of piecoelectric crystals were implanted within the subendocardium of the LAD and LCCA perfusion territories to measure regional contractile function. A catheter was placed in the left atrial appendage for injection of radioactive microspheres to measure regional myocardial perfusion. Bolus injections of adenosine were administered daily via the left atrium to evaluate LAD and LCCA coronary reserve. After stenosis by the Ameroid constrictor, radioactive microspheres were administered to compare regional perfusion within normal myocardium to flow in myocardium supplied by the occluded or stenotic coronary arteries.

Johns and Olson (1954) described a method of experimental **myocardial infarction by coronary occlusion in small animals**, such as **mouse**, **hamster**, **rat** and **guinea pig**.

Sakai et al. (1981) described an **experimental model of angina pectoris in the intact anesthetized rat**. In anesthetized rats the tip of a special carotid cannula was placed closely to the right and left coronary ostium. Single intra-aortic injections of methacholine or acetylcholine (in the presence of physostigmine) developed a reproducible elevation of the ST segment and the T wave of the electrocardiogram. Coronary drugs were tested to prevent these changes.

Gomoll and Lekich (1990) tested the **ferret** for a myocardial ischemia/salvage model. Varying combinations of duration of left anterior descending coronary occlusion and reperfusion were evaluated.

Klein et al. (1995) used intact **pigs** and found myocardial protection by Na^+/H^+ exchange inhibition in ischemic reperfused hearts.

Klein et al. (1997) measured the time delay of cell death by Na^+/H^+ exchange inhibition in regionally ischemic, reperfused **porcine** hearts.

Garcia-Dorado et al. (1997) determined the effect of Na^+/H^+ exchange blockade in ischemic rigor contracture and reperfusion-induced hypercontracture in *pigs* submitted to 55 min of coronary occlusion and 5 h reperfusion. Myocardial segment length analysis with ultrasonic microcristals was used to detect ischemic rigor (reduction in passive segment length change) and hypercontracture (reduction in end-diastolic length).

Symons et al. (1998) tested the attenuation of regional dysfunction in response to 25 cycles of ischemia (2 min) and reperfusion (8 min) of the left circumflex coronary artery in **conscious swine** after administration of a Na^+/H^+ exchange inhibitor. The animals were instrumented to measure arterial blood pressure, regional myocardial blood flow (colored microspheres), systolic wall thickening in the normally perfused left anterior descending and left circumflex coronary artery regions (sonomicrometry), left circumflex coronary artery blood flow velocity (Doppler) and reversibility to occlude the left circumflex coronary artery (hydraulic occluder).

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A.3.2.4
Acute ischemia by injection of microspheres in dogs

PURPOSE AND RATIONALE

Severe left ventricular failure is induced by repeated injections of 50 µm plastic microspheres into the left main coronary artery of anesthetized dogs. Hemodynamic measurements are performed under theses conditions testing drugs which potentially improve cardiac performance. The test can be used to evaluate the influence of drugs on myocardial performance during acute ischemic left ventricular failure in dogs.

PROCEDURE

Dogs of either sex weighing approximately 30 kg are anesthetized by an intravenous bolus injection of 35–40 mg/kg pentobarbital sodium continued by an infusion of 4 mg/kg/h. The animals are placed in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator and controlled by measuring end-expiratory CO_2 concentration as well as blood gases. Two peripheral veins are cannulated for the administration of narcotic (brachial vein) and test compounds (saphenous vein). The ECG is recorded continuously in lead II (Einthoven).

Preparation for hemodynamic measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of the right femoral vein is connected to a pressure transducer (Statham P 23 DB). For determination of left ventricular pressure (LVP), a Millar microtip catheter (Gould PC 350) is inserted via the left carotid artery. Left ventricular enddiastolic pressure (LVEDP) is measured on a high-sensitivity scale. From the pressure curve, *dp*/*dt* max is differentiated and heart rate (HR) is counted. To measure right ventricular pressure, a Millar microtip catheter is inserted via the right femoral vein. Systolic, diastolic and mean pulmonary artery pressure (PAP), mean pulmonary capillary pressure, and cardiac output are measured by a thermodilution technique using a Cardiac Index Computer (Gould SP 1435) and a balloon-tip triple lumen catheter (Gould SP 5105, 5F) with the thermistor positioned in the pulmonary artery via the jugular vein.

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal space, the pericard is opened and the left circumflex coronary artery (LCX) is exposed. To measure coronary blood flow, an electromagnetic flow probe (Hellige Recomed) is placed on the proximal part of the LCX.

Polystyrol microspheres (3M Company, St. Paul, Minnesota, USA) with a diameter of 52.5 ±2.24 µm

are diluted with dextran 70, 60 mg/ml and saline at a concentration of 1 mg microspheres/ml (1 mg = approx. 12 000 beads). For administration of microspheres, an angiogram catheter (Judkins-Schmidt Femoral-Torque, William Cook, Europe Aps. BP 7) is inserted into the left ostium via the left femoral artery.

Induction of failure

The microspheres are injected through the angiogram catheter into the left ostium initially as 10 ml and later as 5 ml boluses about 5 min apart. The microsphere injections produce stepwise elevations of LVEDP. Embolization is terminated when LVEDP has increased to 16–18 mm Hg and/or PAPm has increased to 20 mm Hg and/or heart rate has reached 200 beats/min. The embolization is completed in about 70 min and by injection of an average dose of 3–5 mg/kg microspheres. Hemodynamic variables are allowed to stabilize after coronary embolization for at least 30 min.

Experimental course

The test substance or the vehicle (controls) is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application.

Recordings are obtained

- before embolization
- after embolization
- before administration of test compound
- 5, 30, 45, 60, 90, 120 and, eventually, 150 and 180 min following administration of test drug. At the end of the experiment, the animal is sacrificed by an overdose of pentobarbital sodium.

EVALUATION

Besides the different directly measured hemodynamic parameters, the following data are calculated according to the respective formula:

stroke volume [ml/s],

$$SV = \frac{\text{cardiac output}}{\text{heart rate}}$$

tension index [mm Hg s],

$$IT = \frac{BPs \times \text{heart rate}}{1\,000}$$

Coronary vascular resistance [mm Hg min/ml],

$$CVR = \frac{BPm \times RAPm}{CBF}$$

total peripheral resistance [dyn s/cm^5],

$$TPR = \frac{BPm - RAPm}{\text{cardiac output}} \times 79.9$$

Pulmonary artery resistance [dyn s/cm^5],

$$PAR = \frac{PAPm - PCPm}{\text{cardiac output}} \times 79.9$$

right ventricle work [kg m/min],

$$\text{RVW} = (PAPm - RAPm) \times \text{cardiac output} \times 0.0136$$

left ventricle work [kg m/min],

$$LVW = (BPm - LVEDP) \times \text{cardiac output} \times 0.0136$$

left ventricular myocardial oxygen consumption [ml O_2/min/100 g],

$$MVO_2 = K_1(BPs \times HR) + K_2 \frac{(0.8BPs + 0.2BPd) \times HR \times SV}{BW} + 1.43$$

K_1 = 4.08×10^{-4}
K_2 = 3.25×10^{-4}
BPs = systolic blood pressure [mm Hg]
BPd = diastolic blood pressure [mm Hg]
BPm = mean blood pressure [mm Hg]
CBF = coronary blood flow in left circumflex coronary artery [ml/min]
RAPm = mean right atrial pressure [mm Hg]
PAPm = mean blood pressure A. pulmonalis [mm Hg]
PCPm = mean pulmonary capillary pressure
HR = heart rate [beats/min]
SV = stroke volume [ml]
BW = body weight [kg]

Changes of parameters in drug-treated animals are compared to vehicle controls; statistical significance of the differences is calculated with the Student's *t*-test.

Mean embolization times, doses of microspheres and number of microsphere applications are evaluated.

MODIFICATIONS OF THE METHOD

Gorodetskaya et al. (1990) described a simple method to produce acute heart failure by coronary vessel embolization with microspheres in rats.

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A.3.2.5 Influence on myocardial preconditioning

PURPOSE AND RATIONALE

Damage to the mammalian heart produced by prolonged ischemia and reperfusion can be reduced by "preconditioning" the myocardium via a brief cycle of ischemia and reperfusion prior to the protracted ischemic event. Ischemic preconditioning has been shown to decrease infarct size and increase recovery of post-ischemic ventricular function (Murray et al. 1986), and to reduce leakage of cellular marker proteins indicative for cardiac myocyte death (Volovsek et al. 1992). In addition, preliminary preconditioning also attenuates cardiac arrhythmia associated with subsequent occlusion and reperfusion (Vegh et al. 1990).

The mechanistic basis of this phenomenon is under discussion (Parratt 1994; Parratt and Vegh 1994). Adenosine receptor involvement in myocardial protection after ischemic preconditioning in rabbits has been shown by Baxter et al. (1994). Adenosine (A_1 receptor) antagonists have been demonstrated to block the protection produced by preconditioning (Liu et al. 1991), and short term administration of adenosine was shown to simulate the protective effects of ischemic preconditioning (Toombs et al. 1993). These observations together suggest that adenosine is generated by the short preconditioning ischemia. Other recent pharmacological studies (Gross and Auchampach 1992; Yao and Gross 1994) indicate the involvement of the ATP-sensitive potassium channel. Recent investigations indicate that an increase of NO production after ACE inhibitors may be a part of the protective mechanism (Linz et al. 1992, 1994). Moreover, the involvement of prostanoids and bradykinin in the preconditioning process has been discussed (Wiemer et al. 1991). Gho et al. (1994) found a limitation of myocardial infarct size in the rat by transient renal ischemia, supporting the hypothesis that the mechanism leading to cardiac protection by ischemic preconditioning may not only reside in the heart itself.

PROCEDURE

New Zealand rabbits of either sex weighing 2.5–3.5 kg are initially anesthetized with an intramuscular injection of ketamine (50 mg/ml) /xylazine (10 mg/ml) solution at a dose of 0.6 ml per kg body weight. A tracheotomy is performed to facilitate artificial respiration. The left external jugular vein is cannulated to permit a constant infusion (0.15–0.25 ml/min) of xylazine (2 mg/ml in heparinized saline) to assist in maintaining anesthesia and fluid volume. Anesthesia is also maintained by i.m. injections (0.4–0.6 ml) of ketamine (80 mg/ml) and xylazine (5 mg/ml) solution. After the xylazine infusion is started, animals are respired with room air at a tidal volume of 10 ml/kg and a frequency of 30 inflations per min (Harvard Apparatus, USA). Thereafter, ventilation is adjusted or inspiratory room air is supplemented (5% CO_2/95% O_2) to maintain arterial blood chemistry within the following ranges: pH 7.35–7.45, P_{CO2} 25–45 mm Hg, P_{O2} 90–135 mm Hg. The right femoral artery and vein are isolated and catheterized for measurement of arterial pressure and administration of drugs, respectively.

A thoracotomy is performed in the fourth intercostal space, and the lungs are retracted to expose the heart. The pericardium is cut to expose the left ventricle, and a solid-state pressure transducer catheter (e.g., MicroTip 3F, Millar Instruments, Houston, USA) is inserted through an apical incision and secured to enable measurement of pulsatile left ventricular pressure. The maximal rate of increase in left ventricular pressure (LV*dP*/*dt* max) is determined by electronic differentiation of the left ventricular pressure wave form. A segment of 4-0 prolene suture is looped loosely around a marginal branch of the left main coronary artery to facilitate coronary occlusion during the experiment. Needle electrodes are inserted subcutaneously in a lead II configuration to enable recording of an ECG in order to determine heart rate and help confirm the occurrence of ischemia (ST segment elevation) and reperfusion of the myocardium distal to the coronary occlusion. Continuous recording of pulsatile pressure, ECG, heart rate, and LV*dP*/*dt* are simultaneously displayed on a polygraph (e.g. Gould chart recorder, Gould Inc., Valley View, USA) and digitized in real time by a personal computer. Hemodynamic data are condensed for summary and later statistical analysis.

Ischemic preconditioning is induced by tightening the prolene loop around the coronary artery for 5 min and then loosening to reperfuse the affected myocardium for 10 min prior to a subsequent 30 min occlusion. After surgical preparation, and prior to 30 min of occlusion, rabbits are randomly selected to receive

ischemic preconditioning, no preconditioning, or ischemic preconditioning plus treatment with test drugs. After 30 min of occlusion, the ligature is released and followed by 120 min of reperfusion. Occlusion is verified by epicardial cyanosis distal to the suture, which is usually accompanied by alterations in hemodynamics and ECG. Reperfusion is validated by return of original color. Systemic hemodynamics are summarized for each experimental period. The experiment is terminated after 120 min of reperfusion, and the heart is excised for determinations of infarct size and area at risk.

Immediately before the animal is sacrificed, the marginal branch of the left coronary artery is reoccluded and India ink is rapidly injected by syringe with a 18-g needle into the left ventricular chamber to demarcate blackened normal myocardium from unstained area at risk. After the rabbits are sacrificed, the heart is removed and sectioned in a breadloaf fashion from apex to base perpendicular to the long axis. The right ventricle is removed from each slice leaving only the left ventricle and septum. After each slice is weighed, the portions are washed and incubated in a phosphate buffered saline solution of triphenyl tetrazolium chloride (1 g/ml, Sigma) for 10–15 min. Salvaged myocardium in the area at risk stains brick red, whereas infarcted tissue remains unaltered in color. Slices are then placed between sheets of Plexiglas and the areas (normal, risk, infarct) of each slice are traced on a sheet of clear acetate. Traces are then digitized and analyzed using computerized planimetry to compare the relative composition of each slice with respect to normal tissue, area at risk, and infarcted myocardium. Planimetry is performed with a computerized analysis system, e.g., Quantimet 570C image analysis system (Leica, Deerfield, USA).

Surface areas of normal tissue, area at risk, and infarcted myocardium on both sides of each slide are averaged for the individual slide. The contribution of each slide to total infarcted and area at risk (%) and area at risk as a percentage of total left ventricular mass for the entire left ventricle is prorated by the weight of each slice (Garcia-Dorado et al. 1987). By adding the adjusted contributions from each slice to infarcted tissue, area at risk, and left ventricular mass, a three-dimensional mathematical representation of total myocardial infarct size and risk zone can be calculated for each rabbit, and a mean tabulated for each treatment group for statistical comparison.

EVALUATION

All data are presented as mean ±SD. Systemic hemodynamic data are analyzed by ANOVA using Statistica/W software. Means are considered significantly different at $p < 0.05$.

MODIFICATIONS OF THE METHOD

Li et al. (1990) found in dog experiments that preconditioning with one brief ischemic interval is as effective as preconditioning with multiple ischemic periods.

In contrast, Vegh et al. (1990) found in other dog experiments that two brief preconditioning periods of coronary occlusion, with an adequate period of reperfusion between, reduce the severity of arrhythmias.

Yang et al. (1996) found a second window of protection after ischemic preconditioning in conscious rabbits which minimizes both infarction and arrhythmias.

Late preconditioning against myocardial stunning in conscious pigs together with an increase of heat stress protein (HSP) 70 was described Sun et al. (1995).

Szilvassy et al. (1994) described the anti-ischemic effect induced by ventricular overdrive pacing as a conscious rabbit model of preconditioning. Rabbits were equipped with right ventricular electrode catheters for pacing and intracavital recording and polyethylene cannulae in the left ventricle and right carotid artery to measure intraventricular pressure and blood pressure. One week after surgery in conscious animals, ventricular overdrive pacing at 500 beats/min over 2, 5, or 10 min resulted in an intracavital S-T segment elevation, shortening of ventricular effective refractory period, decrease in maximum rate of pressure development and blood pressure, and increase in left ventricular end-diastolic pressure proportional to the duration of stimulus. A 5-min preconditioning ventricular overdrive pacing applied 5 or 30 min before a 10-min ventricular overdrive pacing markedly attenuated ischemic changes, whereas a 2-min ventricular overdrive pacing had no effect.

The ventricular overdrive pacing induced preconditioning effect was lost in atherosclerotic rabbits (Szilvassy et al. 1995), however, delayed cardiac protection could be induced in these animals (Szekeres et al. 1997).

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A.3.3
Ex vivo methods

A.3.3.1
Plastic casts from coronary vasculature bed

PURPOSE AND RATIONALE

Prolonged administration of coronary drugs has been shown to increase the number and size of interarterial collaterals of dogs and pigs after coronary occlusion (Vineberg et al. 1962; Meesmann and Bachmann 1966). An increased rate of development of collateral arteries was observed after physical exercise in dogs (Schaper et al. 1965), as well as after chronic administration of coronary dilating drugs (Lumb and Hardy 1963). An even more effective stimulus for collateral development is an acute or gradual occlusion of one or several major coronary branches. Filling the arterial coronary bed with a plastic provides the possibility to make the collaterals visible and to quantify them (Schmidt and Schmier 1966; Kadatz 1969).

PROCEDURE

Dogs weighing 10–15 kg are anesthetized with pentobarbital sodium 30 mg/kg i.v. They are respirated artificially and the thorax is opened. After opening of the pericard, Ameroid® cuffs are placed around major coronary branches. Gradual swelling of the plastic material occludes the lumen within 3–4 weeks. The dogs are treated daily with the test drug or placebo. After 1 week recovery period they are submitted to exercise on a treadmill ergometer. After 6 weeks treatment, the animals are sacrificed, the heart removed and the coronary bed flushed with saline. The liquid plastic Araldite® is used to fill the whole coronary tree from the bulbus aortae. The aortic valves are glued together in order to prevent filling of the left ventricle. Red colored Araldite® is used to fill the arterial tree. The venous part of the coronary vasculature can be filled

with blue colored Araldite® from the venous sinus. The uniformity of the filling pressure, the filling time, and the viscosity of the material are important. Polymerization is complete after several hours. Then, the tissue is digested with 35% potassium hydroxide. The method gives stable preparations which can be preserved for a long time.

EVALUATION

Plastic casts from drug treated animals are compared with casts from dogs submitted to the same procedure without drug treatment.

CRITICAL ASSESSMENT OF THE METHOD

The procedure allows impressive demonstration of the formation of arterial collaterals. The results of post mortem Araldit® impletion agree with the functional results of experimental coronary occlusion.

MODIFICATIONS OF THE METHOD

Boor and Reynolds (1977) described a simple planimetric method for determination of left ventricular mass and necrotic myocardial mass in postmortem hearts.

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A.4
Calcium uptake inhibiting activity

A.4.0.1
General considerations

Cellular calcium flux is regulated by receptor-operated and voltage-dependent channels which are sensitive to inhibition by calcium entry blockers. The term calcium antagonist was introduced by Fleckenstein (1964, 1967) when two drugs, prenylamine and verapamil, originally found as coronary dilators in the LANGENDORFF-experiment, were shown to mimic the cardiac effects of simple Ca^{2+}-withdrawal, diminishing Ca^{2+}-dependent high energy phosphate utilization, contractile force, and oxygen requirement of the beating heart without impairing the Na^+-dependent action potential parameters. These effects were clearly distinguishable from β-receptor blockade and could promptly be neutralized by elevated Ca^{2+}, β-adrenergic catecholamines, or cardiac glycosides, measures that restore the Ca^{2+} supply to the contractile system. In the following years many Ca^{2+}-antagonists were introduced to therapy. Specific Ca^{2+}-antagonists interfere with the uptake of Ca^{2+} into the myocardium and prevent myocardial necrotization arising from deleterious intracellular Ca^{2+} overload. They act basically as specific inhibitors of the slow transsarcolemnal Ca^{2+} influx but do not or only slightly affect the fast Na^+ current that initiates normal myocardial excitation.

Calcium channels and the sites of action of drugs modifying channel function have been classified (Bean 1989; Porzig 1990; Tsien and Tsien 1990; Spedding and Paoletti 1992). Four main types of voltage dependent calcium channels are described:

1. L type (for long lasting),
2. T type (for transient),
3. N type (for neuronal), and
4. P type (for Purkinje cells).

They differ not only by their function (Dolphin 1991) and localization in tissues and cells but also by their sensitivity to pharmacological agents (Ferrante and Triggle 1990; Dascal 1990; Kitamura et al. 1997) and by their specificity to radioligands.

The widely distributed L-type channels exist in isoforms (L1, 2, 3, 4) and consist of several subunits, known as α_1, α_2, β, γ, δ. They are sensitive to dihydropyridines, phenylalkylamines or benzothia-zepines, but insensitive to ω-conotoxin and ω-agatoxin. The segments required for antagonist binding have been analyzed (Peterson et al. (1996, Schuster et al. 1996; Mit-

terdorfer et al. 1996; Hockerman et al. 1997; Striessnig et al. 1998; Catterall 1998).

The T-type channels are located mainly in the cardiac sinoatrial node and have different electrophysiological characteristics from L-type channels (Massie 1997; Perez-Reyes et al. 1998).

N- and P-type calcium channels blockers occur in neuronal cells and are involved in neurotransmitter release (Olivera et al. 1987; Bertolino and Llinás 1992; Mintz et al. 1992; Woppmann et al. 1994; Diversé-Pierluissi et al. 1995; Miljanich and Ramachandran 1995; Fisher and Bourque 1996; Ikeda 1996; Ertel et al. 1997; Sinnegger et al. 1997).

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A.4.1 *In vitro* methods

A.4.1.1 ^{3}H-nitrendipine binding *in vitro*

PURPOSE AND RATIONALE

Radiolabeled dihydropyridine calcium channel antagonists such as ^{3}H-nitrendipine are selective ligands for a drug receptor site associated with the voltage-dependent calcium channel. A constant concentration of the radioligand ^{3}H-nitrendipine (0.3–0.4 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of plasma membranes from bovine cerebral cortices. If the test drug exhibits any affinity to calcium channels, it is able to compete with the radioligand for channel binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent is the test drug.

PROCEDURE

Materials and solutions:

Preparation buffer	Tris-HCl pH 7.4	50 mM
Incubation buffer	Tris-HCl Genapol pH 7.4	50 mM 0.001%
Radioligand	^{3}H-nitrendipine Specific activity 2.59–3.22 TBq/mmol (70–87 Ci/mmol) (NEN)	
For inhibition of ^{3}H-ni-trendipine binding in non-specific binding experiments	Nifedipine (Sigma)	

Two freshly-slaughtered bovine brains are obtained from the slaughter house and placed in ice-cold preparation buffer. In the laboratory, approx. 5 g wet weight of the two frontal cerebral cortices are separated from the brains.

Membrane preparation

The tissue is homogenized (glass Teflon potter) in ice-cold preparation buffer, corresponding to 1 g cerebral wet weight/50 ml buffer, and centrifuged at 48 000 *g* (4 °C) for 10 min. The resulting pellets are resuspended in approx. 270 ml preparation buffer, and the homogenate is centrifuged as before. The final pellets are dissolved in preparation buffer, corresponding to 1 g cerebral cortex wet weight/30 ml buffer. The membrane suspension is immediately stored in aliquots of 5–10 ml at –77 °C. Protein content of the membrane suspension is determined according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed and centrifuged at 48 000 *g* (4 °C) for 10 min. The resulting pellets are resuspended in a volume of ice-cold incubation buffer, yielding a membrane suspension with a protein content of 0.6–0.8 mg/ml. After homogenization (glass Teflon potter), the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental course

As 1,4-dihydropyridines tend to bind to plastic material, all dilution steps are done in glass tubes.

For each concentration samples are prepared in triplicate. The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation experiments

total binding:

- 50 µl ^{3}H-nitrendipine (12 concentrations, 5×10^{-11}–4×10^{-9} M)
- 50 µl incubation buffer

non-specific-binding:

- 50 µl ^{3}H-nitrendipine (4 concentrations, 5×10^{-11}–4×10^{-9} M)
- 50 µl nifedipine (5×10^{-7} M)

Competition experiments

- 50 µl ^{3}H-nitrendipine (1 constant concentration, 3–4×10^{-10} M)
- 50 µl incubation buffer without or with non-labeled test drug (15 concentrations, 10^{-10}–10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (0.6–0.8 mg protein/ml). The samples are incubated for 60 min in a bath shaker at 25 °C. The reaction is stopped by subjecting the total incubation volume to rapid vacuum filtration over glass fibre filters. Thereby the membrane-bound is separated from the free radioactivity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a Packard liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding – non-specific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-nitrendipine versus non-labeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D{}^3\mathrm{H} \times IC_{50}}{K_D{}^3\mathrm{H} + [^3\mathrm{H}]}$$

IC_{50} = concentration of the test drug, which displaces 50% of specifically bound ^{3}H-nitrendipine in the competition experiment

$[^3\mathrm{H}]$ = concentration of ^{3}H-nitrendipine in the competition experiment.

$K_D{}^3\mathrm{H}$ = dissociation constant of ^{3}H-nitrendipine, determined from the saturation experiment.

The K_i-value of the test drug is the concentration, at which 50% of the receptors are occupied by the test drug.

The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

Standard data:

- nifedipine $K_i = 2$–4×10^{-9} mol/l

MODIFICATIONS OF THE METHOD

Several other calcium entry blockers, such as nimodipine, diltiazem, verapamil and desmethoxyverapamil, have been labelled and used for binding studies in order to elucidate the calcium channel recognition sites and may be used for further classification of calcium antagonists (Ferry and Glossmann 1982; Glossmann et al. 1983; Goll et al. 1984; Lee et al. 1984; Glossmann et al. 1985; Schoemaker and Langer 1985; Ruth et al. 1985; Reynolds et al. 1986).

Tissue heterogeneity of calcium channel antagonist binding sites has been demonstrated by Gould et al. (1983).

Photoaffinity labeling of the cardiac calcium channel with 1,4-dihydropyridine(–)-[3H]azidopine was described by Ferry at al (1987).

Binding sites for ω-conotoxin appear to be primarily associated with the N-type of voltage-dependent calcium channels (Feigenbaum et al. 1988; Wagner et al. 1988).

Cohen et al. (1992) recommended the peptide ω-agatoxin IIIA as a valuable pharmacological tool being the only known ligand that blocks L-type calcium channels with high affinity at all voltages and causes, unlike the 1,4-dihydropyridines, no block of T-type calcium channels.

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A.4.2
Isolated organs

A.4.2.1
Calcium antagonism on action potential of isolated guinea pig papillary muscle

PURPOSE AND RATIONALE

Intracellular action potential in the guinea pig papillary muscle is recorded. Partial depolarization is achieved by potassium enriched Ringer solution and by addition of isoproterenol. Resting potential is increased to 40 mV resulting in inactivation of the fast sodium channel. Under these conditions, upstroke velocity is an indicator for calcium flux through the membrane, which is decreased by calcium blockers.

PROCEDURE

Guinea pigs of either sex (Pirbright White strain) weighing 300–400 g are sacrificed by stunning, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed, placed in a container of prewarmed, pre-oxygenated Ringer solution, and the pericardium and the atria are trimmed away. The left ventricle is opened and the two strongest papillary muscles removed. They are fixed between a suction electrode for electrical stimulation and a force transducer for registration of contractions. Initially, normal Ringer solution oxygenated with carbogen (95% O_2/5% CO_2) at a temperature of 36 °C is used. A standard micro electrode technique is applied to measure the action potential via a glass micro elec-

trode containing 3 M KCl solution, which is inserted intracellularly. The papillary muscle is stimulated with rectangular pulses of 1 V and of 1 ms duration at intervals of 500 ms. The interval between two stimuli is variable in order to determine refractory periods. The intensity of the electrical current is just below the stimulation threshold. The intracellular action potential is amplified, differentiated for registration of upstroke velocity (Hugo Sachs micro electrode amplifier), together with the contraction force displayed on an oscilloscope (Gould digital storage oscilloscope OS 4000), and recorded (Gould 2400 recorder).

After an incubation period of 30 min the Ringer solution is changed to the following composition containing 5 times more potassium and 10% less sodium.

- NaCl 8,1 g/L
- KCl 1,0 g/L
- $CaCl_2$ 0,2 g/L
- $NaHCO_3$ 0,1 g/L
- glucose 5,0 g/L

For further depolarization, isoproterenol (1.0 mg per 100 ml) is added. By this measure, resting potential is increased to about 40 mV, resulting in inactivation of the fast inward sodium channel. The resulting slow rising action potential is sensitive to calcium antagonistic drugs (Kohlhardt and Fleckenstein 1977).

The test compound is added at a concentration of 1 µg/ml. Effective compounds are tested at lower concentrations and compared with the standard (nifedipin at concentrations of 0.01 and 0.1 µg/ml)

EVALUATION

The decrease of upstroke velocity is tested at various concentrations of the test compound and compared with the standard.

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A.4.2.2 Calcium antagonism in the isolated guinea pig atrium

PURPOSE AND RATIONALE

k-Strophanthine (and other cardiac glycosides) inhibit the membrane-bound Na^+/K^+-activated ATP-ase which leads to an increase in intracellular Ca^{2+}-concentration. Ca^{2+} ions activate the contractile apparatus, causing a distinctive enhancement of contractions. The procedure can be used to evaluate a compound's calcium channel blocking activity by measuring its ability to decrease atrial contractions induced by k-strophanthine.

PROCEDURE

Apparatus

HSE-stimulator 1 (Hugo Sachs Elektronik, D-79232 March-Hugstetten, Germany)

Stimulation data:

- frequency 1.5 Hz
- duration 3 ms
- voltage 3–8 V

Experiment

Guinea pigs of either sex weighing 200–500 g are sacrificed with a blow to the nape of the neck and exsanguinated. The left atrium is removed, placed in an organ bath and attached to an isotonic strain gauge, its base being wired to an electrode of the stimulator. The Ringer-solution is aerated with carbogen and kept at 36 °C. The atrium is continuously stimulated via stimulator 1, the voltage being slowly increased up to the threshold level. Contractions are recorded on a polygraph. Prior to drug administration, two prevalues are obtained by adding 2 µg/ml k-strophanthine-α (Cymarin) to the organ bath and measuring the increase in contractile force. Following a 15 min washout and recovery period, the test drug is added to the bath followed by administration of k-strophanthine-α 10 min later. The change in contractile force is always measured 10 min after the addition of k-strophanthine-α.

Standard compounds:

- Verapamil hydrochloride
- Nifedipine

EVALUATION

The percent inhibition of k-strophanthine-α induced contraction is determined.

MODIFICATIONS OF THE METHOD

Calcium antagonists can also be evaluated in the LANGENDORFF heart preparation (Lindner and Ruppert 1982).

Leboeuf et al. (1992) reported the protective effect of bepridil and flunarizine against veratrine-induced contracture in rat atria concluding from the results in this model that these agents may be more effective as L-type calcium ion-channel blockers in protecting against calcium overload during ischaemia and reperfusion injury.

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A.4.2.3 Calcium antagonism in the isolated rabbit aorta

PURPOSE AND RATIONALE

Contraction of aorta rings is induced by adding potassium chloride or norepinephrine to the organ bath containing slightly modified Krebs bicarbonate buffer. Test drugs with calcium channel blocking activity have a relaxing effect.

PROCEDURE

Rabbits of either sex weighing 3–4 kg are sacrificed with an overdose of pentobarbital sodium. The chest cavity is opened and the descending thoracic aorta (from the level of the aortic arch to the level of the diaphragm) is rapidly removed and placed in a beaker of oxygenated Krebs bicarbonate buffer at 37 °C.

The content of magnesium and calcium is slightly diminished in the Krebs bicarbonate buffer resulting in the following composition:

• NaCl	118.4	mMol
• KCl	4.7	mMol
• KH_2PO_4	1.2	mMol
• $MgSO_4 \cdot 2\,H_2O$	1.2	mMol
• $CaCl_2 \cdot 2\,H_2O$	1.9	mMol
• $NaHCO_3$	25.0	mMol
• dextrose	10.0	mMol
• EDTA	0.013	mMol

The tissue is then transferred to a dish containing fresh oxygenated, warmed Krebs solution. Fat and loose connective tissue are carefully removed while keeping the tissue moist with the solution. Eight rings of 4–5 mm width are obtained and each is mounted in a 20 ml tissue bath which contains the oxygenated warmed Krebs solution. Initial tension is set at 1.0 g. The tissue is allowed to incubate over a period of 2 h, during which time the Krebs solution is changed every 15 min. Also during this time, tension is maintained at 1.0 g. Just prior to the end of the 2 h equilibration period, the Krebs solution is changed again and the tissue is allowed to stabilize at 1.0 g tension. A sustained contraction is then generated by addition of either 40 mM KCl or 2.9×10^{-3} mM norepinephrine.

Twenty min after addition of the agonist, the test drug is added so that the final concentration in the bath is 1×10^{-5} M. The percent relaxation reading is taken 30 min after addition of the test drug. If at least 30% relaxation occurs, an accumulative concentration-relaxation curve is established. There is a 30 min period of time between the addition of each concentration of test compound.

EVALUATION

Active tension is calculated for the tissue at the time point just prior to the addition of the test compound and also at the point 30 min after the addition of each concentration of test compound. Active tension is defined as the difference between the generated tension and the baseline tension. The percent relaxation from the predrug, precontracted level is calculated for each concentration of test compound. A number of 5 experiments constitutes a dose range. An ID_{50} is calculated by linear regression analysis.

MODIFICATIONS OF THE METHOD

Hof and Vuorela (1983) compared three methods for assessing calcium antagonism on rabbit aorta smooth muscle.

Matsuo et al. (1989) reported a simple and specific screening method for Ca-entry blockers. In the presence of various Ca-channel blockers, 1×10^{-4} M Ca^{2+} causes relaxation of rat uterine smooth muscle that has been tonically contracted with oxytocin in calcium-free medium after prolonged preincubation with 3 mM EGTA.

Micheli et al. (1990) used spirally cut preparations of rat aorta and rings of rabbit ear artery to test calcium entry blocker activity.

Rüegg et al. (1985) described a smooth muscle cell line originating from fetal rat aorta to be suitable for the study of voltage sensitive calcium channels. Calcium channel antagonists inhibited both the basal and the potassium chloride stimulated $^{45}Ca^{2+}$ uptake.

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A.4.2.4 Calcium antagonism in the isolated guinea pig pulmonary artery

PURPOSE AND RATIONALE

Contraction of the pulmonary artery is induced by changing the normal Tyrode solution in the organ bath against a potassium enriched solution. This contraction can be inhibited by calcium blockers.

PROCEDURE

The following solutions are used:

	Normal Tyrode solution [mMol]	Potassium enriched Tyrode solution [mMol]
NaCl	135.0	89.0
KCl	3.7	50.0
$MgSO_4$	0.81	0.81
NaH_2PO_4	0.41	0.41
$NaHCO_3$	11.0	11.0
$CaCl_2$	2.25	2.25
Glucose	5.6	5.6

Guinea pigs (Pirbright White strain) of either sex weighing 400–500 g are sacrificed by stunning. The pulmonary artery is removed and cut spirally at an angle of 45°. The resulting strip is cut to lengths of 2 cm and one piece is suspended in oxygenated normal Tyrode solution in an organ bath at 37 °C with a preload of 1 g. Contractions are registered with an isotonic strain transducer and recorded on a polygraph.

After 1 h equilibrium time, normal solution is exchanged with potassium enriched Tyrode solution. The artery strip reacts with a contraction which achieves after 10 min 90–95% of its maximum. After an additional 10 min, exchange to normal Tyrode solution is performed. Ten min later again a contraction is induced by potassium enriched solution. When the height of the contraction has reached a constant level, the test substance is added and again potassium induced contraction recorded. The height of the contraction is expressed as percent of initial potassium induced contraction.

After lavage, the procedure is repeated with a higher dose or the standard.

EVALUATION

For calculation of a regression line, the decrease of contraction versus control after various doses is measured in mm. The percentage of inhibition after various doses is taken for calculation of an ED_{50}.

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A.4.3 *In vivo* methods

A.4.3.1 Evaluation of calcium blockers in the pithed rat

PURPOSE AND RATIONALE

Using the cardioaccelerator response in pithed rats, calcium entry blockers can be distinguished from other agents which have modes of action not involving direct blockade of calcium entry (Clapham 1988).

PROCEDURE

Male Sprague-Dawley rats (250–350 g) are anaesthetized with methohexitone sodium (50 mg/kg i.p.). Following cannulation of the trachea, the rats are pithed through one orbit with a stainless steel rod and immediately artificially respired with room air (78 strokes/min, 1 ml/100 g body weight) via a Palmer small animal respiration pump. A jugular vein is cannulated for administration of drugs. Arterial blood pressure is recorded from a carotid artery using a pressure transducer. Heart rate is derived from the phasic arterial pressure signal with a phase lock loop ratemeter (BRL Instrument Services). Both parameters are displayed on a recorder. The animals are kept warm by an incandescent lamp positioned about 25 cm above them. The pithing rod is withdrawn so that the tip lays in the thoracic portion of the spinal cord. All rats then receive (+)tubocurarine (1.5 mg/kg i.v.) and are bilaterally vagotomized.

The cardioaccelerator response is obtained by continuous electrical stimulation of the thoracic spinal cord with square wave pulses of 0.5 ms duration, at supramaximal voltage at a frequency of 0.5 Hz using the pithing rod as a stimulating electrode. An indifferent electrode is inserted subcutaneously in the femoral region. Only rats with a resulting tachycardia of more than 100 beats/min are included into the experiments.

When the cardioaccelerator response has stabilized for about 3–5 min, cumulative intravenous doses of drug or corresponding vehicle are administered. Succeeding doses are given when the response to the previous dose has stabilized.

Calcium antagonists and β-blockers inhibit dose-dependent the tachycardia elicited by electrical stimulation of the spinal cord, whereas lignocaine and nicorandil are not effective.

Doses of β-blockers or calcium-antagonists, which reduce the tachycardia to 50% are tested again. Three min after administration of the drug, calcium gluconate (1 mg/min) or water (0.1 ml/min) are infused using a Harvard apparatus compact infusion pump. The effects of calcium entry blockers, but not of β-adrenoreceptor blockers, are antagonized.

EVALUATION

The level of tachycardia immediately prior to drug administration is taken as 100% and responses to drugs are expressed as a percentage of this predose tachycardia. If an inhibitory effect >50% is seen, then an ID_{50} (with 95% confidence limits) is interpolated from linear regression analysis. Significance of differences between the groups receiving calcium gluconate and their parallel vehicle controls is calculated by Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

Differentiation between the effects of β-blockers and calcium-antagonists can be achieved in a relatively simple *in vivo* model.

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A.5 Anti-arrhythmic activity

A.5.0.1 General considerations

Guidelines for the study of arrhythmias in man and animals regarding the experimental design as well as the classification, quantification, and analysis were given as the Lambeth Conventions by Walker et al. (1988).

Classification

Anti-arrhythmic drugs have been classified into various groups and subgroups (Vaughan Williams 1970, 1975, 1984, 1988, 1991, 1992; Borchard et al. 1989; Frumin et al. 1989; Harumi et al. 1989; Colatsky and Follmer 1990; Podrid 1990; Coromilas 1991; Nattel 1991; Rosen and Schwartz 1991; Scholz 1991; Woosley 1991; Ravens 1992; Sanguinetti 1992; Grant 1992; Nattel 1993; Scholz 1994). This classification is based on electrophysiological effects (e.g., action potential) and on interaction with membrane receptors and ion channels. The heterogeneity of classification criteria resulted in vivid discussions (The Sicilian Gambit 1991; Vaughan Williams 1991, 1992). In particular, a clinical study (CAST investigators 1990) challenged the therapeutic value of some anti-arrhythmic drugs.

Weirich and Antoni (1990, 1991) proposed a subdivision of class-1-anti-arrhythmic drugs according to the saturation behavior of frequency-dependent block and its onset-kinetics.

Class I anti-arrhythmic drugs directly alter membrane conductance of cations, particularly those of Na^+ and K^+. They reduce upstroke velocity, V_{max}, of the cardiac action potential by blockade of the fast Na^+ channel. This leads to a depression of conduction velocity, a prolongation of the voltage- and time-dependent refractory period and an increase in the threshold of excitability in cardiac muscle. Class I anti-arrhythmic drugs are subclassified according their effect on the action potential duration.

Class I A anti-arrhythmic drugs (Quinidine-like substances, e.g., disopyramide, procainamide, ajmaline) lengthen the action potential duration which is reflected in the ECG as lengthening of the QT-interval. This effect is added to that on fast sodium channel resulting in delayed recovery from inactivation.

Class I B anti-arrhythmic drugs (lidoacaine-like drugs, e.g., mexiletine, phenytoin, tocainide), in contrast, shorten the action potential duration.

Class I C anti-arrhythmic drugs (e.g., encainide, flecainide, propafenone, indecainide) produce quinidine- and lidocaine-like effects and exert differential actions on the duration of action potential in Purkinje fibres (shortening) and ventricular muscle.

Class II anti-arrhythmic drugs are β-adrenergic antagonists. They exert their anti-arrhythmic effects by antagonizing the electrophysiological effects of catecholamines which are mainly mediated by an increase in slow calcium inward current.

Class III anti-arrhythmic drugs (e.g., amiodarone, bretylium, sotalol) prolong the action potential and lead to a corresponding increase in the effective refractory period. The action is mainly due to a block of outward repolarizing currents. However, activation of sodium and calcium inward currents that prolong the plateau of the action potential may also be involved.

Class IV anti-arrhythmic drugs (e.g., verapamil, diltiazem) are slow calcium channel blockers suppressing the slow calcium inward current and calcium-dependent slow action potentials.

Experimentally induced arrhythmias

Winslow (1984) reviewed the methods for the detection and assessment of antiarrhythmic activity.

Szekeres (1979) suggested a rational screening program for the selection of effective antiarrhythmic drugs.

Arrhythmia models in the rat were reviewed by Cheung et al. (1993).

Arrhythmogenic stimuli can be divided into three groups: chemical, electrical and mechanical (Szekeres and Papp 1975; Wilson 1984).

Chemically induced arrhythmias

A large number of chemical agents alone or in combination are capable of inducing arrhythmias. Administration of anesthetics like chloroform, ether, halothane (sensitizing agents) followed by a precipitating stimulus, such as intravenous adrenaline, or cardiac glycosides (usually ouabain), aconitine, and veratrum alkaloids cause arrhythmias. The sensitivity to these arrhythmogenic substances differs among various species.

Electrically induced arrhythmias

The possibilities to produce arrhythmias by electrical stimulation of the heart and the difficulties for evaluation of anti-arrhythmic drugs by this approach have been discussed by Szekeres (1971). Serial electrical stimulation result in flutter and fibrillation and it is possible to reproduce some of the main types of arrhythmias of clinical importance. The flutter threshold or the ventricular multiple response threshold may be determined in anesthetized dogs before or after the administration of the test drug.

Mechanically induced arrhythmias

Arrhythmias can be induced directly by ischemia or by reperfusion. After ischemia either by infarction or by coronary ligation several phases of arrhythmias are found. The two stage coronary artery ligation technique described by Harris (1950) focuses on late arrhythmias.

Curtis and Walker (1988) examined seven scores in an attempt to validate the use of arrhythmia scores in an *in vivo* model of conscious rats.

The influence on reperfusion arrhythmias can be tested in various species, e g., rat, pig, dog and cat (Bergey et al. 1982; Winslow 1984; Curtis et al. 1987; Brooks et al. 1989).

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A.5.0.2 Electrocardiography in animals

PURPOSE AND RATIONALE

Recording of the electrocardiogram is an essential tool in the evaluation of anti-arrhythmic drugs (Johnston et al. 1983; Curtis and Walker 1986; Adaikan et al. 1992). Similar to the heart rate, the electrocardiogram is different between various species (Bazett 1920; Kisch 1953; Heise and Kimbel 1955; Beinfield and Lehr 1968; Budden et al. 1981; Driscoll 1981; Osborn 1981; Hayes et al. 1994). Many authors used the bipolar lead II between right foreleg and left hindleg, which is in line with the neutrally placed heart. Additionally, lead I (between right and left foreleg) stated to lie in the axis of the horizontal heart, and lead III (between left foreleg and left hindleg) in line with the vertical heart, may be used as well as unipolar leads (usually designed as V_1 to V_6) and the unipolar leads designed as aVL, aVR, and aVF. Out of several species being used the procedure for rats (Penz et al. 1992; Hayes et al. 1994) is described.

PROCEDURE

Male Sprague Dawley rats weighing 250–300 g are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbitone. The right jugular vein is cannulated for injections, while the left coronary artery is cannulated for recording blood pressure on a polygraph. The ECG is recorded using a Lead type II of configuration along the anatomical axis of the heart as determined by palpation. ECGs are recorded at a standard chart speed of 100 mm/s on a polygraph and simultaneously on a storage oscilloscope. Measurements of intervals are made on the chart recorder and from the memory trace of the monitor.

Since in the rat it is difficult to detect a T-wave that corresponds exactly with the T-wave seen in other species (Beinfeld and Lehr 1968; Driscoll 1981; Surawicz 1987) T-wave calculations are made on the basis of the repolarization wave that follows the QRS complex. The following variables are measured:

σT = time for the depolarization wave to cross the atria, *P-R* interval, *QRS* interval, *Q-T* interval, and *RSh* (the height between the peak of R and S wave). The *RSh* magnitude is taken as a measure of the extent of S-wave depression as exerted by class I sodium channel blocking antiarrhythmics.

EVALUATION

Statistical analyses are based on ANOVA followed by Duncan's test for differences of means. In order to demonstrate the relationships between and drug effects, standard cumulative dose-response curves are constructed.

MODIFICATIONS OF THE METHOD

Osborne (1973, 1981) described a restraining device facilitating electrocardiogram recording in **conscious rats**.

Curtis and Walker (1986), Johnston et al. (1983) studied the responses to ligation of a coronary artery and the actions of antiarrhythmics in conscious rats.

Hayes et al. (1994) studied the ECG in **guinea pigs**, rabbits and primates.

Stark et al. (1989) described an epicardic surface and stimulation technique (SST-ECG) in Langendorff perfused guinea pig hearts.

Epicardial His bundle recordings in the guinea pig *in vivo* were described by Todt and Raberger (1992).

Chronic recording from the His bundle in awake nonsedated **dogs** was reported by Karpawich et al. (1983) and by Atlee et al. (1984).

Van de Water et al. (1989) reported a formula to correct the QT interval of the electrocardiogram in dogs for changes in heart rate.

Wu et al. (1990) described a dual electrophysiologic test for atrial anti-reentry and ventricular antifibrillatory studies in anesthetized dogs. The reentry portion of the model was created surgically by a Y-shaped crushing around the tissue between the superior and inferior vena cava and tissue parallel to the AV groove. The pacing induced tachycardia that results from circus movements around the tricuspid ring was very persistent in duration and regular in cycle length. The antifibrillatory activities were assessed by determination of the ventricular fibrillation threshold using a train-stimuli method.

Weissenburger et al. (1991) developed an experimental model of the long QT syndrome in conscious dogs for screening the bradycardia-dependent proarrhythmic effects of drugs and for studying the electrophysiology of "torsades de pointes".

Holter monitoring in conscious dogs was described by Krumpl et al. (1989a,b).

Coker (1989) recommended the anesthetized **rabbit** as a model for ischemia- and reperfusion-induced arrhythmias.

Baboons and **monkeys** *(Macaca sp.)* were used by Adaikan et al. (1992).

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A.5.0.3 Aconitine antagonism in rats

PURPOSE AND RATIONALE

The plant alkaloid aconitine persistently activates sodium channels. Infusion of aconitine in the anesthetized rat causes ventricular arrhythmias. Drugs considered to have anti-arrhythmic properties can be tested in aconitine-intoxicated rats.

PROCEDURE

Male Ivanovas rats weighing 300–400 g are used. The animals are anesthetized by intraperitoneal injection of 1.25 g/kg urethane. Five µg/kg aconitine dissolved in 0.1 N HNO_3 is administered by continuous infusion into the saphenous vein of 0.1 ml/min and the ECG in lead II is recorded every 30 seconds. The test compound is injected orally or intravenously at a screening dose of 3 mg/kg 5 min before the start of the aconitine infusion. Eight–ten animals are used per compound.

EVALUATION

The anti-arrhythmic effect of a test compound is measured by the amount of aconitine/100 g animal (duration of infusion) which induces

- ventricular extrasystoles
- ventricular tachycardia
- ventricular fibrillation
- and death.

Higher doses of aconitine in the treated group as compared to an untreated control group are an indication of anti-arrhythmic activity.

Statistical significance between the groups is assessed by the Student's *t*-test.

The scores are allotted for the intensity and the duration of the effect relative to the efficacy of standard compounds.

Standard data:

- Procainamid, 5 mg/kg i.v. and lidocaine, 5 mg/kg, i.v. lead to an increase in LD_{100} by 65% (corresponds to LD_{100} of approximately 9 µg/100 g).

CRITICAL ASSESSMENT OF THE METHOD

Aconitine – antagonism *in vivo* has been proven as a valuable screening method for anti-arrhythmic activity.

MODIFICATIONS OF THE METHOD

Scherf (1947) studied the auricular tachycardia caused by aconitine administration in **dogs**.

Scherf et al. (1960) provoked atrial flutter and fibrillation in anesthetized dogs by application of a few crystals of aconitine or delphinine to the surface of the right atrium in the appendix area near the head of the sinus node.

Mc Leod and Reynold (1962) induced arrhythmia by aconitine in the isolated **rabbit** atrium.

Nwangwu et al. (1977) used aconitine as arrhythmogenic agent for screening of anti-arrhythmic agents in **mice**.

Yamamoto et al. (1993) used urethane-anesthetized rats under artificial respiration with tubocurarine pretreatment. After thoracotomy and incision of the pericardium, a piece of filter paper soaked with aconitine solution was applied to the right atrium. Test drugs were applied by continuous i.v. infusion. In addition to ECG lead II, intra-atrial ECG was monitored.

Aconitine-antagonism in conscious mice as screening procedure has been recommended by Dadkar and Bhattacharya (1974) and in anesthetized mice by Winslow (1980).

Nakayama et al. (1971) described the topical application of aconitine in a small cup placed on the right atrium of dogs to induce supraventricular arrhythmias.

A method using the **cat** has been developed by Winslow (1981).

Other arrhythmogenic agents

In addition to the aconitine model Vaille et al. (1992) demonstrated the selectivity of a $CaCl_2$ continuous infusion screening method in rats for the evaluation of antiarrhythmic calcium antagonists.

A mouse chloroform model was recommended by Lawson (1968).

Vargaftig et al. (1969) induced ventricular fibrillation in mice by inhalation of chloroform.

Papp et al. (1967) proposed the experimental $BaCl_2$-arrhythmia as a quantitative assay of anti-arrhythmic drugs.

Al-Obaid et al. (1998) used calcium chloride-induced arrhythmias for anti-arrhythmic activity evaluation in anesthetized male rats. Cardiac arrhythmias were induced by a single intravenous injection of 10% $CaCl_2$ (50 mg/kg). The induced arrhythmias were then analyzed for magnitude of initial bradycardia, onset, incidence and duration of the induced fibrillations. After the induction of the arrhythmia, the animal was allowed to recover completely (15–20 min) and the test compound was injected in different doses intravenously. The effect of the test compound on the basal heart rate was then examined and the percentage change in the heart rate was calculated. Seven min later, the arrhythmogenic dose of $CaCl_2$ was re-administered and the effect of the treatment on the induced arrhythmia parameters was evaluated as percentage change in the measured parameters or as protection or non-protections against the induced fibrillations.

Tripathi and Thomas (1986) described a method for the production of ventricular tachycardia in the rat and guinea pig by exposing the animals to benzene vapors for 2 min followed by an intravenous adrenaline injection.

Arrhythmias could be induced by changing the medium of cultured rat heart muscle cells (Wenzel and Kloeppel 1978).

In isolated rat hearts ventricular fibrillation was induced by isoprenaline and a catechol-O-methyl transferase inhibitor at high perfusion temperature (Sono et al. 1985).

Takei (1994) described experimental arrhythmia in guinea pigs induced by grayanotoxin-I, a biologically active diterpenoid from the plant family of Ericaceae.

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A.5.0.4
Digoxin-induced ventricular arrhythmias in anesthetized guinea pigs

PURPOSE AND RATIONALE

Overdose of cardiac glycosides, such as digoxin, induces ventricular extrasystoles, ventricular fibrillation, and finally death. The occurrence of these symptoms can be delayed by anti-arrhythmic drugs.

PROCEDURE

Male guinea pigs (Marioth strain) weighing 350–500 g are anesthetized with 35 mg/kg pentobarbital sodium intraperitoneally. Trachea, one jugular vein and one carotid artery are catheterized. Positive pressure ventilation is applied with a respiratory pump (Rhema GmbH, Germany) at 45 breaths/min. The carotid artery is used for monitoring systemic blood pressure via a pressure transducer. Digoxin is infused into the jugular vein with a perfusion pump (ASID BONZ PP 50) at a rate of 85 µg/kg in 0.266 ml/min until cardiac arrest. The electrocardiogram (lead III) is recorded with subcutaneous steel-needle electrodes (Hellige 19).

Treated groups ($n = 5–10$ animals) receive the test drug either orally 1 h or intravenously 1 min prior to the infusion. The control group (n = at least 5 animals) receives the digoxin infusion only. The period until the onset of ventricular extrasystoles, ventricular fibrillation, and cardiac arrest is recorded. The total amount of infused digoxin (µg/kg) to induce ventricular fibrillation is calculated. Standard drugs are lidocaine (3 mg/kg i.v.) or ramipril (1 mg/kg p.o.).

EVALUATION

Using Student's *t*-test the doses of digoxin needed to induce ventricular extrasystoles, or ventricular fibrillation, or cardiac arrest, respectively, after treatment with anti-arrhythmic drugs are compared statistically with controls receiving digoxin only.

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A.5.0.5
Strophanthin or ouabain induced arrhythmia

PURPOSE AND RATIONALE

Acute intoxication with the cardiac glycoside strophanthin K induces ventricular tachycardia and multifocal ventricular arrhythmias in dogs. This can be used as a test model to evaluate the effect of potential anti-arrhythmic drugs on ventricular arrhythmias.

PROCEDURE

Male or female dogs of either sex weighing approximately 20 kg are used. The animals are anesthetized by intravenous injection of 30–40 mg/kg pentobarbital sodium. Two peripheral veins are cannulated for the administration of the arrhythmia-inducing substance (*V. brachialis*) and the test compound (*V. cephalica antebrachii*). For intraduodenal administration of the test drug, the duodenum is cannulated. Electrocardiogram is registered with needle electrodes from lead II. Heart frequency is derived from R-peaks of ECG. Two–three animals are used for one compound.

Strophanthin K is administered by continuous i.v.-infusion at a rate of 3 µg/kg/min. Thirty–fourty min later, signs of cardiac glycoside intoxication appear leading to ventricular tachycardia or to multifocal ventricular arrhythmias. When this state is achieved, the strophanthin infusion is terminated. When the arrhythmias are stable for 10 min, the test substance is administered intravenously in doses between 1.0 and 5.0 mg/kg or intraduodenally in doses between 10 and 30 mg/kg.

ECG II recordings are obtained at times: –0.5, 1, 2, 5 and 10 min following administration of test drug.

For i.v. administration: A test compound is considered to have an anti-arrhythmic effect if the extrasystoles immediately disappear. If the test compound does not show a positive effect, increasing doses are administered at 15 min-intervals. If the test substance does reverse arrhythmias, the next dose is administered after the reappearance of stable arrhythmias.

For i.d. administration: A test compound is considered to have a definite anti-arrhythmic effect if the extrasystoles disappear within 15 min. The test drug is considered to have "no effect" if it does not improve strophanthin intoxication within 60 min following drug administration.

EVALUATION

Evaluation of the therapeutic effect of a drug is difficult and somewhat arbitrary since there is no clear-cut correlation between effectiveness of a test compound and duration of its effect, i.e. return to normal ECGs. The standard drugs ajmaline, quinidine and lidocaine re-establish normal sinus rhythm at doses of 1 and 3 mg/kg (i.v.) and 10 mg/kg (i.d.). Arrhythmias are eliminated for 20 min (i.v.) and for >60 min (i.d.) following drug administration.

MODIFICATIONS OF THE METHOD

Ettinger et al. (1969) used arrhythmias in dogs induced by ouabain to study the effects of phentolamine in arrhythmia.

Garrett et al. (1964) studied the antiarrhythmic activity of *N,N*-diisopropyl-*N*'-diethylaminoethylurea hydrochloride in anesthetized dogs with arrhythmias induced by ouabain, aconitine or acetylcholine. Furthermore, ultra-low frequency ballistocardiograms with ECG registration were performed in dogs.

Raper and Wale (1968) studied the effects on ouabain- and adrenaline-induced arrhythmias in **cats**.

Kerr et al. (1985) studied the effects of a vasodilator drug on ouabain-induced arrhythmias in anesthetized dogs.

A modified method for the production of cardiac arrhythmias by ouabain in anesthetized cats was published by Rao et al. (1988).

Brooks et al. (1989) infused ouabain intravenously to **guinea pigs** and determined the onset of ventricular extrasystoles and of fibrillation.

Thomas and Tripathi (1986) studied the effects of α-adrenoreceptor agonists and antagonists with different affinity for α_1- and α_2-receptors on ouabain-induced arrhythmias and cardiac arrest in guinea pigs.

Krzeminski (1991) and Wascher et al. (1991) used of ouabain-induced arrhythmia in guinea pigs for the evaluation of potential antidysrhythmic agents.

Al-Obaid et al. (1998) used ouabain-induced arrhythmias in anesthetized **Wistar rats** for evaluation of cyclopenteno[b]thiophene derivatives as antiarrhythmic agents.

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A.5.0.6 Ventricular fibrillation electrical threshold

PURPOSE AND RATIONALE

The use of anti-arrhythmic drugs in the treatment of ventricular arrhythmias aims to prevent the development of ventricular fibrillation. Several electrical stimulation techniques have been used to measure ventricular fibrillation threshold such as single pulse stimulation, train of pulses stimulation, continuous 50-Hz stimulation and sequential pulse stimulation.

PROCEDURE

Adult dogs weighing 8–12 kg are anesthetized with sodium pentobarbital (35 mg/kg) and ventilated with air using a Harvard respiratory pump. Systolic arterial pressure is monitored and body temperature maintained by a thermal blanket. The chest is opened by a midline sternotomy and the heart suspended in a pericardial cradle. The sinus node is crushed and a 2.0 mm diameter Ag-AgCl stimulating electrode is embedded in a Teflon disc sutured to the anterior surface of the left ventricle. The heart is then driven by 3-ms square anodal constant current pulses for 400 ms of the basic cycle and is prematurely stimulated by one 3-ms test

stimulus through the driving electrode. Electrical stimulation is programmed by a digital stimulator. A recording electrode is placed on the surface of each ventricle. A silver plate is implanted under the skin in the right femoral region as indifferent electrode. Lead II of the body surface electrocardiogram is monitored. To determine ventricular fibrillation threshold (VFT), a 0.2- to 1.8-second train of 50-Hz pulses is delivered 100 ms after every eighteenth basic driving stimulus. The current intensity is increased from the diastolic threshold in increments of 10 μA to 1.0 mA or until ventricular fibrillation occurs. The minimal current intensity of the pulse train required to induce sustained ventricular fibrillation is defined as the VFT. When ventricular fibrillation occurs, the heart is immediately defibrillated and allowed to recover to control conditions for 15 to 20 min. Anti-arrhythmic drugs are administered through the femoral vein.

EVALUATION

Ventricular fibrillation threshold (VFT) is determined before and after administration of test drugs at given time intervals. The mean values of 10 experiments are compared using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Marshall et al. (1981) and Winslow (1984) suggested to determine VFT in the pentobarbitone anesthetized rat.

Wu et al. (1989) recommended a conscious dog model for re-entrant atrial tachycardia.

Wu et al. (1990) described a dual electrophysiologic test for atrial anti-re-entry and ventricular antifibrillatory studies in dogs. The re-entry portion of the model was created surgically by a Y-shaped crushing around the tissue between the superior and inferior vena cava and tissue parallel to the AV groove. The antifibrillatory activities were assessed by determination of the ventricular fibrillation threshold using a train-stimuli method.

A chronically prepared rat model of electrically induced arrhythmias was described by Walker and Beatch (1988).

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A.5.0.7 Coronary artery ligation, reperfusion arrhythmia and infarct size in rats

PURPOSE AND RATIONALE

Coronary artery ligation in anesthetized rats results in arrhythmias and myocardial infarction. Following occlusion of the left main coronary artery, very marked ventricular dysrhythmias occur. Electrocardiogram is recorded during ligation and subsequent reperfusion. The amount of infarcted tissue is measured by means of p-nitro-blue tetrazolium chloride-staining in myocardial sections. The model is used to test drugs with potential anti-arrhythmic activities.

PROCEDURE

Groups of 8–10 male Sprague-Dawley rats weighing 350–400 g are used. The animals are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. The trachea is intubated to allow artificial ventilation (Starling pump). A catheter is placed in an external jugular vein for administration of test compounds. Peripheral blood pressure is recorded from the common carotid artery using a pressure transducer and a polygraph. The chest is opened by left thoracotomy at the forth intercostal space. After opening the pericard, the heart is exteriorized by gentle pressure on the chest walls and a thin silk thread (Ethicon 1.5 metric, 4-0) attached to an atraumatic needle is placed around the left coronary artery about 2–3 mm distal of the origin of the left coronary artery for later ligation. From that point on, the animal is ventilated with room air using a stroke volume of 1 ml/100 g body

weight at a rate of 54 strokes/min. The heart is then placed back in the chest cavity. Any animal in which this procedure itself produces dysrhythmias or a sustained fall in mean arterial blood pressure to less than 70 mm Hg has to be discarded from the study.

After an equilibration time of approx. 45 min, the test substance or the vehicle (control) is administered by intravenous injection. Five min later, the ligature at the left coronary artery is closed either for 15 or 90 min (in case infarct-size is assessed) and subsequently reperfused for 30 min. For oral application, the test compounds are dissolved or suspended in the vehicle 30 min before occlusion. Peripheral blood pressure and ECG lead II are recorded continuously during the whole experiment. Rectal temperature is maintained at 38 °C. The numbers of ventricular premature beats (VPB), ventricular tachycardia (VT) and ventricular fibrillation (VF) are counted in the occlusion and reperfusion periods and evaluated according to the guidelines of the Lambeth Convention (Walker et al. 1988).

Preparation to determine infarct size

At the end of the reperfusion period, the animal is sacrificed with an overdose of pentobarbital sodium, the heart is dissected and cut into transversal sections (approx. 1 mm thick) from the apex to the base. The slices are stained with p-nitro-blue tetrazolium chloride solution (0.25 g/L p-nitroblue-tetrazolium chloride in Sörensen phosphate buffer, containing 100 mM D, L-maleate) in order to visualize the infarct tissue (blue/violet-stained healthy tissue, unstained necrotic tissue). The slices are photographed on color transparency film for the determination of infarct area. Left ventricle and infarct area are measured by planimetry from projections of all slices with the exclusion of the apex and the slice containing the ligature.

EVALUATION

The following parameters are evaluated:

- mortality
- hemodynamics
  - peripheral blood pressure [mm Hg]
  - heart rate [beats/min]
  - pressure rate index (PRI) (BPs × HR) [mm Hg × beats/1 000]
- arrhythmias
  - ventricular extrasystoles (= premature ventricular contractions) (PVC)
    - percent animals with PVC
    - number of PVC/5 or 30 min
  - ventricular tachycardia (VT) (VT defined as any run of seven or more consecutive ventricular extrasystoles)
    - percent animals with VT
    - duration [s] of VT/5 or 30 min
  - ventricular fibrillation (VF)
    - percent animals with VF
    - duration [s] of VF/5 or 30 min
- infarct size (area)

The different characteristics are evaluated separately and compared with a positive control (5 mg/kg nicainoprol i.v.).

Changes of parameters in drug-treated animals are compared to vehicle control values.

Statistical significance is assessed by the Student's *t*-test.

MODIFICATIONS OF THE METHOD

Leprán et al. (1983) placed a loose silk loop around the left coronary artery and passed the thread through a cylinder shaped polyethylene tube outside the thorax. The rats were allowed to recover from primary surgery. The loose ligature was tightened 7–10 days thereafter and arrhythmias recorded by ECG tracings.

Johnston et al. (1983) described the responses to ligation of a coronary artery in conscious rats and the actions of anti-arrhythmics.

As reported in Sect. A.3.1.2 and A.3.1.3, the isolated heart according to LANGENDORFF and the isolated working rat heart preparation can be used for ligation experiments inducing arrhythmias. Lubbe et al. (1978) reported ventricular arrhythmias associated with coronary artery occlusion and reperfusion in the isolated perfused rat heart as a model for assessment of antifibrillatory action of anti-arrhythmic agents.

Bernier et al. (1986) described reperfusion-induced arrhythmias in the isolated perfused rat heart. The isolated rat heart was perfused according the LANGENDORFF-technique. A ligature was placed around the left anterior descending coronary artery close to its origin. The arterial occlusion was maintained for 10 min followed by reperfusion. Test compounds were included in the perfusion medium. With epicardial ECG-electrodes the number of premature ventricular complexes, the incidence and duration of ventricular fibrillation, and the incidence of ventricular tachycardia were recorded.

Abraham et al. (1989) tested antiarrhythmic properties of tetrodotoxin against occlusion-induced arrhythmias produced by ligation of the left anterior descending coronary artery in the rat.

MacLeod et al. (1989) tested a long acting analogue of verapamil for its actions against arrhythmias induced by ischemia and reperfusion in conscious and anesthetized rats, as well as for effects on epicardial intracellular action potentials.

Aye et al. (1997) tested the effects of a Na^+/H^+ exchange inhibitor on reperfusion ventricular arrhythmias in rat hearts.

Ferrara et al. (1990) studied the effect of flecainide acetate on reperfusion- and barium-induced ventricular tachyarrhythmias in the isolated perfused rat heart by monitoring heart rate, coronary flow rate, left ventricular systolic pressure, dp/dt_{max}, and the voltage of the epicardial electrogram.

Heterogeneity of ventricular remodeling after acute myocardial infarction in rats has been reported by Capasso et al. (1992).

Bellemin-Baurreau et al. (1994) described an *in vitro* method for evaluation of antiarrhythmic and antiischemic agents by using programmed electrical stimulation of the isolated rabbit heart after ligation of the left ventricular branch of the coronary artery and a reperfusion period of 15 min.

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Black and Rodger (1996), Black (2000) reviewed the methods used to study experimental myocardial ischemic and reperfusion injury in various animal species.

Linz et al. (1997) reported that in isolated rat hearts with ischemia-reperfusion injuries, perfusion with bradykinin reduces the duration and incidence of ventricular fibrillations, improves cardiodynamics, reduces release of cytosolic enzyme, end preserves energy-rich phosphate and glycogen stores.

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A.5.0.8
Ventricular arrhythmia after coronary occlusion

A.5.0.8.1
Ventricular fibrillation after coronary occlusion and reperfusion in anesthetized dogs

PURPOSE AND RATIONALE

Coronary artery occlusion in anesthetized dogs is accompanied by an increase in heart rate, heart contractility, left ventricular end-diastolic pressure, and blood pressure as well as by ventricular arrhythmias. During a subsequent reperfusion period, a high percentage of control animals die from ventricular fibrillation. Drugs with potential protective effects are tested which reduce both hemodynamic and electrical changes.

PROCEDURE

Dogs of either sex weighing 20–25 kg are used. Anesthesia is induced by intravenous injection of 30 mg/kg thiobutobarbital sodium and maintained by i.v. administration of 20 mg/kg chloralose and 250 mg/kg urethane followed by subcutaneous administration of 2 mg/kg morphine. The animals are placed in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator (Bird Mark 7). A peripheral vein (saphenous vein) is cannulated for the administration of test compound. The ECG is recorded continuously in lead II (Einthoven).

Preparation for hemodynamic measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of a femoral artery is connected to a pressure transducer (Statham P 23 DB). For determination of left ventricular pressure (LVP), a Millar microtip catheter (PC 350) is inserted via the left carotid artery. Left ventricular end-diastolic pressure (LVEDP) is measured on a high-sensitivity scale; heart rate (HR) is determined from the LVP wave form. Myocardial contractility is measured as the rate of rise of LVP (dp/dt max). The sum of ST-segment elevations is calculated from five values of the peripheral limbs in ECG lead II. The pressure-rate index ($PRI = BPs \times HR$) serves as a measure of oxygen consumption.

Experimental course

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal space, the pericard is opened and the left anterior descending coronary artery (LAD) is prepared. A silk suture is placed around the LAD, just below the first diagonal branch. After an equilibration period of approx. 45 min., the test substance or the vehicle (controls) is administered as an intravenous bolus. Twenty min later, the ligature at the coronary artery is closed for 90 min. During the occlusion period, the test compound or the vehicle (controls) are given by continuous infusion. After release of the coronary obstruction, the animal is monitored for a 30 min reperfusion period. All parameters are recorded during the whole experiment. At the end of the test, surviving animals are sacrificed by an overdose of pentobarbital sodium.

EVALUATION

The following parameters are evaluated:

- mortality
- hemodynamics
- arrhythmias
  - ventricular extrasystoles (= premature ventricular contractions) (PVC)
  - percent animals with PVC

- number of PVC/5 or 30 min
- ventricular tachycardia (VT) (VT defined as any sequence of seven or more consecutive ventricular extrasystoles)
- duration [s] of VT/5 or 30 min
- ventricular fibrillation (VF)
- percent animals with VF

The different characteristics are evaluated separately. Changes of parameters in drug-treated animals are compared to vehicle controls. Statistical significance of the differences is calculated by means of the Student's *t*-test.

Standard data:
Mortality: In an representative experiment, 10 out of 12 of control animals died from ventricular fibrillation during the 30 min reperfusion period. One out of 8 molsidomine-treated animals died and the death was also from ventricular fibrillation during the reperfusion phase. (Molsidomine was given as a continuous infusion of 0.5 mg/kg/ml/min during the occlusion period; controls received saline).

MODIFICATIONS OF THE METHOD

Varma and Melville (1963) described ventricular fibrillation induced by coronary occlusion during hypothermia in **dogs**.

Wilkerson and Downey (1978) described a technique for producing ventricular arrhythmias in dogs through coronary occlusion by an embolus (glass beads) being introduced into the coronary circulation via a rigid cannula which is inserted through the carotid artery.

Weissenburger et al. (1991) described a model in dogs suitable for screening the bradycardia-dependent proarrhythmic effects of drugs and for studying the electrophysiology of "torsades de pointes".

Coker (1989) recommended the anesthetized **rabbit** as a model for ischemia- and reperfusion-induced arrhythmias.

Thiemermann et al. (1989) described a rabbit model of experimental myocardial ischemia and reperfusion. Drugs were administered by intravenous infusion 5 min after the occlusion of the left anterior-lateral coronary artery and continued during the 60 min occlusion and subsequent 3 h reperfusion periods.

Barrett et al. (1997) described a method of recording epicardial monophasic action potentials and ischemia-induced arrhythmias following coronary artery ligation in intact rabbits.

Naslund et al. (1992) described a closed chest model in *pigs*. Occlusion was induced in pentobarbitone anesthetized, mechanically ventilated **pigs** by injection of a 2 mm ball into a preselected coronary artery. Reperfusion was achieved by retraction of the ball via an attached filament.

D'Alonzo et al. (1994) evaluated the effects of potassium channel openers on pacing- and ischemia-induced ventricular fibrillation in anesthetized pigs.

Premaratne et al. (1995) used a **baboon** open chest model of myocardial ischemia and reperfusion. Baboons underwent occlusion of the left anterior descending coronary artery for 2 h. Fifteen min after occlusion, the treated group received hyaluronidase i.v. over a 10-min period. The ischemic period was followed by 22 h of reperfusion. At the end of the reperfusion period, the hearts were excised and the perfusion bed at risk for infarction was determined by infusion of a microvascular dye.

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A.5.0.8.2 Harris dog model of ventricular tachycardia

PURPOSE AND RATIONALE

In 1950, Harris found that the mortality in dogs after coronary occlusion with a 2-stage ligation procedure was lower than with 1-stage ligation. The left descending coronary artery is partially occluded for 30 min after which time total ligation is performed. Under these conditions arrhythmias develop within 4–7 h, reach a peak between 24 and 48 h and abate within 3–5 days.

PROCEDURE

Surgical procedure

Dogs of either sex are anesthetized by intravenous injection of methohexitone sodium (10 mg/kg), an endotracheal tube is inserted, and anesthesia maintained with halothane. The heart is exposed through an incision in the fourth or fifth intercostal space. The anterior descending branch of the left coronary artery is dissected free below its second branch and ligated in two stages. Two ligatures are placed around the artery and a 21 gauge needle. The first ligature is tied round the artery and the needle, which is then removed. Thirty min later, the second ligature is tied tightly round the artery. The chest is closed in layers 30 min after the second ligature has been tied, and the dog is allowed to recover.

Test procedure

Further observations are made when the dogs are conscious, e.g., 22–24 h after ligation of the coronary artery. The dogs are positioned to lie on their side and remain in this position throughout the experiment. Mean blood pressure is recorded from a catheter placed in the femoral artery. Lead II and aV_L of the electrocardiogram and blood pressure are continuously recorded for a control period of 30 min before and during drug administration. Drugs are administered either by injection or by continuous infusion via a hind leg vein.

EVALUATION

The number of sinus and ectopic beats are counted for each successive 5-min period. Beats with a distinct P wave preceding a mean frontal QRS vector of normal duration are counted as sinus in origin; all others are denoted as ectopic.

MODIFICATIONS OF THE METHOD

The model which resembles late arrhythmias occurring in postinfarction patients has been used with modifications by many authors (e.g., Kerr et al. 1985; Reynolds and Brown 1986; Gomoll 1987; Garthwaite et al. 1989; Krumpl et al. 1989a,b; Trolese-Mongheal et al. 1985, 1991; Spinelli et al. 1991).

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A.5.0.8.3 Protection against sudden coronary death

PURPOSE AND RATIONALE

The group of Lucchesi described an experimental dog model to test protection against sudden coronary death (Patterson et al. 1982; Uprichard et al. 1989a,b; Chi et al. 1990a,b, 1991; Kitzen et al. 1990; Black et al. 1991, 1993).

Surgical preparation

Purpose-bred male mongrel dogs weighing 14–22 kg are anesthetized with 30 mg/kg pentobarbital i.v. The dogs are ventilated with room air through a cuffed endotracheal tube and a Harvard respirator. A cannula is inserted in the left external jugular vein. A left thoracotomy is performed between the fourth and fifth ribs, and the heart is exposed and suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) is isolated at the tip of the left atrial appendage, and the left circumflex coronary artery (LCX) is isolated ~1 cm from its origin. After a 20-gauge hypodermic needle has been placed on the LAD, a ligature is tied around the artery and the needle. The needle is then removed, resulting in critical stenosis of the vessel. The LAD is perfused for 5 min in the presence of the critical stenosis. Ischemic injury of the anterior ventricular myocardium is achieved by 2-h occlusion of the LAD by a silicon rubber snare. The vessel is reperfused after 2 h in the presence of the critical stenosis. During the period of LAD reperfusion, an epicardial bipolar electrode (1-mm silver posts, 3-mm inter-electrode separation) is sutured on the left atrial appendage for subsequent atrial pacing. A bipolar plunge electrode (25-gauge stainless steel, 5 mm long, 3 mm separation) is sutured on the interventricular septum, adjacent to the occlusion site and overlying the right ventricular outflow tract (RVOT). Two similar stainless steel bipolar plunge electrodes are sutured to the left ventricular (LV) wall: one at the distribution of the LAD distal to the occlusion (infarct zone, IZ), and the second in the distribution of the LCX (non-infarct zone, NZ). A 30-gauge silver-coated copper wire electrode is passed through the wall and into the lumen of the LCX and sutured to the adjacent surface of the heart. Silver disc electrodes are implanted subcutaneously for ECG monitoring. The surgical incision is closed and the animals are allowed to recover.

Drug treatment

The animals are treated after the recovery period during the 3 days of programmed electrical stimulation either with the test drug or with the solvent.

Electrophysiologic studies and programmed electrical stimulation

Programmed electrical stimulation (PES) is performed between days 3 and 5 after induction of anterior myocardial infarction by occlusion/perfusion of the LAD. Animals are studied while conscious and unsedated. Heart rate, ECG intervals and other electrophysiologic parameters (for details see original publications) are determined before PES is started. Premature ventricular stimuli are introduced in the region of the right ventricular outflow tract. The extra stimuli are triggered from the R-wave of the ECG, and the R-S_2 coupling interval is decreased from 350 ms until ventricular refractoriness occurs. At this time, double and triple ventricular extra stimuli are introduced during sinus rhythm. Ventricular tachyarrhythmias are defined as 'non- sustained', if five or more repetitive ventricular responses are initiated reproducibly, but terminated spontaneously. Ventricular tachyarrhythmias are defined as 'sustained', if they persist for at least 30 s or, in the event of hemodynamic compromise, require ventricular burst pacing for their termination.

Sudden cardiac death

A direct anodal 15 μA current from a 9-V nickel-cadmium battery is passed through a 250 Ohm resistor and applied to the electrode in the lumen of the left circumflex coronary artery. The cathode of the battery is connected to a s.c. implanted disc electrode. Lead II ECG is recorded for 30 s every 15 min on a cardiocassette recorder. After 24 h of constant anodal current or development of ventricular fibrillation, the animals are sacrificed, the hearts are excised and the thrombus mass in the LCX is removed and weighed. The heart is sectioned transversely and incubated for 15 min at 37 °C in a 0.4% solution of tetrazolium triphenyl chloride for identification of infarcted areas. Time of onset of ventricular ectopy and of lethal arrhythmia is provided from recordings of the cardiocassette.

EVALUATION

Non-sustained and sustained tachyarrhythmias are evaluated.

CRITICAL ASSESSMENT OF THE METHOD

Sudden coronary death is one of the leading causes of death in developed countries. These facts warrant the use of complicated models in higher animals for search of active drugs.

MODIFICATIONS OF THE METHOD

Cahn and Cervoni (1990) reviewed of the use of animal models of sudden cardiac death for drug development.

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A.5.0.8.4 Ventricular fibrillation induced by cardial ischemia during exercise

PURPOSE AND RATIONALE

Billman and his group developed methods to evaluate antiarrhythmic drugs for their activity in cardiovascular parameters in an exercise-plus-ischemia test.

PROCEDURE

Surgical preparation

Mongrel dogs, weighing 15.4 to 19.1 kg, are anesthetized and instrumented to measure left circumflex CBF, left ventricular pressure and ventricular electrogram (Billman and Hamlin 1996; Billman et al. 1993, 1997; Schwartz et al. 1984). The animals are given Innovar Vet (0.02 mg/kg fentanyl citrate and 1 mg/kg hydroperidol i.v.) as a pre-anesthetic, whereas a surgical plane of anesthesia is induced with sodium pentobarbital (10 mg/kg i.v.). A left thoracotomy is made in the fourth intercostal space, and the heart is exposed and supported by a pericardial cradle. A 20-MHz pulsed Doppler flow transducer and a hydraulic occluder are placed around the left circumflex artery. A pair of insulated silver-coated wires are sutured to the epicardial surface of both the left and right ventricles. These electrodes are used for ventricular pacing or to record a ventricular electrogram from which HR is determined using a Gould Biotachometer (Gould Instruments, Cleveland, OH). A pre-calibrated solid-state pressure transducer (Konigsberg Instruments, Pasadena, CA) is inserted into the left ventricle via a stab wound in the apical dimple. Finally, a two-stage occlusion of the left anterior descending coronary artery is performed approximately one third the distance from the origin to induce an anterior wall myocardial infarction. This vessel is partially occluded for 20 min and then tied off. All leads from the cardiovascular instrumentation are tunneled under the skin to exit on the back of the animal's neck. A transdermal fentanyl patch that delivers 75 µg/h for 72 h is placed on the back of the neck (secured with adhesive tape) to decrease post-operative discomfort. In addition, bupivacaine HCl, a long-acting local anesthetic, is injected to block the intercostal nerves (i.e., pain fibers) in the area of the incision. Each animal is placed on prophylactic antibiotic therapy (amoxicillin 500 mg p.o.) three times daily for 7 days. The animals are placed in an "intensive care" setting for the first 24 h and placed on antiarrhythmic therapy (Billman and Hamlin 1996; Billman et al. 1993, 1997; Schwartz et al. 1984).

Exercise-plus-ischemia test

The studies begin 3 to 4 weeks after the production of the myocardial infarction. The animals are walked on a motor-driven treadmill and trained to lie quietly without restraint on a laboratory table during this recovery period. Susceptibility to VF is then tested. The animals run on a motor-driven treadmill while workload is increased every 3 min for a total of 18 min. The protocol begins with a 3-min warm-up period, during which the animals run at 4.8 km/h at 0% grade. The speed is increased to 6.4 km/h, and the grade is in-

creased every 3 min as follows: 0%, 4%, 8%, 12% and 16%. During the last minute of exercise, the left circumflex coronary artery is occluded, the treadmill is stopped and the occlusion is maintained for 1 additional min (total occlusion time, 2 min). Large metal plates (diameter, 11 cm) are placed across the animal's chest so that electrical defibrillation can be achieved with minimal delay but only after the animal is unconscious (10–20 s after VF begin). The occlusion is immediately released if VF occur.

The animals then receive one or more of the following treatments:

1. the exercise-plus-ischemia test is repeated after pretreatment with the standard drug glibenclamide (1.0 mg/kg i.v.). The drug is injected in a cephalic vein; 3 min before exercise begins.
2. The exercise-plus-ischemia test is repeated after pretreatment with the test drug
3. Finally, a second control (saline) exercise plus ischemia test is performed 1 week after the last drug test. At least 5 days are intermitted between drug treatments. Drugs are given in a random order.

Refractory period determination

On a subsequent day, the effective refractory period is determined using a Medtronic model 5325 programmable stimulator, both at rest and during myocardial ischemia. The heart is paced for 8 beats (S_1; intrastimulus interval, 300 ms; pulse duration, 1.8 ms at twice-diastolic threshold of ~6 mA). The intrastimulus interval is progressively shortened between the last paced beat and a single extra-stimulus (S_2). The refractory period represents the shortest interval capable of generating a cardiac response and is measured using either the left or right ventricular electrodes. This procedure is completed within 30 s. Once the control values are determined, refractory period measurements are repeated after the standard drug glibenclamide (1.0 mg/kg i.v.), or the test drug. After the completion of these studies, refractory period is determined during myocardial ischemia (2-min occlusion of the left circumflex coronary artery) ~60 s after the onset of the coronary occlusion.

Reactive hyperemia studies

The K_{ATP} has been implicated in vascular regulation, particularly CBF (Aversano et al. 1991; Belloni and Hintze 1991; Daut et al. 1990). Therefore, the effects of standard and test drug on the response to brief interruptions in CBF are also evaluated. Animals are placed on a laboratory table, and the left circumflex coronary is occluded three or four times for 15 s. At least 2 min (or until CBF had returned to preocclusion base line) elapse between occlusions. The occlusions are then repeated 5 min after standard and test drug. On the subsequent day, the studies are repeated with the drug that had not been given the previous day.

EVALUATION

All hemodynamic data are recorded on a Gould model 2800S eight-channel recorder (Cleveland, OH) and a Teac model MR-30 FM tape recorder (Tokyo, Japan). Coronary blood flow is measured with a University of Iowa Bioengineering flowmeter model 545 C-4 (Iowa City, IA). The rate of change of left ventricular pressure [$d(LVP)/dt$] is obtained by passing the left ventricular pressure through a Gould differentiator that has a frequency response linear to >300 Hz. The data are averaged over the past 5 s of each exercise level. The coronary occlusion data are averaged over the last 5 s before and at the 60-s line point (or VF onset) after occlusion onset. The total area between the peak CBF and return to base line is measured for each 15-s occlusion, and the percent repayment is calculated. The reactive hyperemia response to each occlusion is then averaged to obtain one value for each animal. The data are then analyzed using analysis of variance for repeated measures. When the F ratio is found to exceed a critical value ($P < 0.05$), Scheffe's test is used to compare the mean values. The effects of the drug intervention on arrhythmia formation are determined using a χ^2 test with Yates' correction for continuity. All data are reported as mean ±SEM. Cardiac arrhythmias, PR interval and QT interval are evaluated at a paper speed of 100 mm/s. QT interval is corrected for HR using Bazett's method.

CRITICAL ASSESSMENT OF THE METHOD

Tests combining coronary constriction with physical exercise may resemble most closely the situation in coronary patients.

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A.5.0.9
Characterization of anti-arrhythmic activity in the isolated right ventricular guinea pig papillary muscle

PURPOSE AND RATIONALE

According to Vaughan-Williams (1970) anti-arrhythmic drugs are divided into 4 different classes depending on their mode of action. Class I anti-arrhythmic agents decrease the upstroke velocity of the action potential through blockade of Na^+ channels. Class II drugs block β-receptors. Class III anti-arrhythmic agents prolong action potential duration, presumably through blockade of K^+ channels. Class IV anti-arrhythmic agents inhibit the slow calcium influx during the plateau of the action potential through Ca^{2+} channel blockade. These electrophysiological actions also have functional manifestations, e.g., Na^+ channel blockade decreases excitability, K^+ channel blockade lengthens refractory period, and Ca^{2+} channel blockade decreases tension of cardiac muscle. A simple and accurate non-microelectrode method is necessary to identify and classify potential anti-arrhythmic drugs into the classes I, III, and IV. In right ventricular guinea pig papillary muscle developed tension (DT), excitability (EX), and effective refractory period (ERP) are measured.

PROCEDURE

Guinea pigs of either sex weighing 200–400 g are stunned, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed, placed into a container of pre-warmed, pre-oxygenated physiologic solution and the pericardium, atria, and other tissues are removed. The heart is then pinned to a dissection dish, and the right ventricle is opened. The tendinous end of the papillary muscle is ligated with a silk thread, and the chordae tendinae are freed from the ventricle. The opposite end of the papillary muscle is then cut free close to the ventricular wall. The non-ligated end of the papillary muscle is clamped into a tissue holder, the end of which is a leucite block containing platinum wire field electrodes.

The preparation is transferred to a tissue bath containing 75 ml of a physiological salt solution that is gassed continuously with 95% O_2/5% CO_2 and maintained at a temperature of 35 °C and a pH of 7.4. The silk thread is used to connect the muscle to a Grass FT03C force transducer. An initial resting tension of 1 g is established. Muscles are field stimulated to contract isometrically. The stimulus duration is 1 ms, the frequency 1 Hz, and the voltage twice threshold. Pulses are delivered with the use of a Grass S88 constant voltage stimulator, and developed tension is recorded with the use of a polygraph recorder. The preparation is equilibrated in this manner for 90 min with bath solution changes every 15 min. Control measurements of the force-frequency curve, stimulus strength-duration curve and the effective refractory period are made following the 75 min bath exchange, i.e., during the last 15 min of equilibration.

The force-frequency curve is obtained by measuring developed tension over a range of stimulation frequencies (0.3, 0.5, 0.8, 1.0, and 1.2 Hz). The tissue is contracted for 90 s at each of these frequencies with a brief period of stimulation at 1.5 Hz inserted between increments. The purpose of the 1.5 Hz insert is to keep "pacing history" constant as well as to minimize progressive, nonspecific depression during the lower frequency stimulation series. Both pre- and postdrug developed tension (at each frequency) are expressed as a percentage of the predrug developed tension at 1 Hz. The percent change in post treatment (versus pretreatment) developed tension at 1 Hz is used to quantitate an agent's inotropic effect.

The stimulus strength-duration curves are determined by varying the stimulus duration (0.1, 0.4, 0.8, 1.0, 1.5, 3.0, and 3.4 ms) and finding the threshold voltage that produced a 1:1 correspondence between stimulus and response at each duration. The degree of shift in the strength-duration curve is measured by computing the area between the pre- and post-treatment curves. The boundaries for the area are determined by the first (x-axis parallel) and the last (x-axis perpendicular) durations and by lines from the origin to the second and fourth durations.

Effective refractory period (ERP) is measured at 1 Hz using twin pulse stimuli. After every 8–10 pulses, a second delayed stimulus (S_2) identical to the basic drive pulse (S_1) is introduced. This procedure is repeated, shortening the delay ($S_1 - S_2$) by 5 ms increments. The value of the ERP is taken as the longest delay ($S_1 - S_2$) for which there is a single response to twin pulses. The change in ERP is computed as the difference (ms) between the pre- and post-treatment

At the conclusion of the 90 min predrug equilibration period, an aliquot of the test drug designed to achieve the desired final concentration is added to the bath. The tissue must equilibrate for 1 h in the drug solution before postdrug measurements of the force-frequency curve, stimulus strength-duration curve, and effective refractory period are obtained.

EVALUATION

The changes in effective refractory period (ERP) (post treatment minus pretreatment), the degree of shift in the strength-duration curve (geometrical area between pre- and post-treatment curves), and the percent changes in post treatment developed tension at 1 Hz are calculated. The results of these calculations are used to classify the compound as a class I, III, or IV anti-arrhythmic agent on the basis of its effect on developed tension, excitability, and effective refractory period. An upward and right shift of the strength-duration curve (decrease in excitability) is characteristic for a class I anti-arrhythmic agent, such disopyramide. Selective prolongation of effective refractory period is characteristic for class III anti-arrhythmic agents, such as sotalol. Depression of developed tension, and/or flattening or reversal of the force-frequency curve is characteristic for a class IV anti-arrhythmic agent, such as verapamil.

CRITICAL ASSESSMENT OF THE METHOD

The model of the electrically stimulated isolated guinea pig papillary muscle is a simple method to classify anti-arrhythmic agents. Some drugs have multiple actions and, therefore, belong in more than one class. For further characterization analysis of the action potential is necessary.

MODIFICATIONS OF THE METHOD

O'Donoghue and Platia (1991) recommended the use of monophasic action potential recordings for the evaluation of anti-arrhythmic drugs.

Shibuya et al. (1993) studied the effects of the local anesthetic bupivacaine on contraction and membrane potential in isolated canine right ventricular papillary muscles. From analysis of action potential it is concluded that at low concentrations contraction is depressed mainly due to a Na^+ channel block, whereas at high concentrations also Ca^{2+} channels may be blocked.

Kodama et al. (1992), Maryuama et al. (1995) studied the effects of potential antiarrhythmics on maximum upstroke velocity and duration of action potential in isolated right papillary muscles of guinea pigs as well as the influence of these agents on single ventricular myocytes.

Borchard et al. (1982) described a method for inducing arrhythmias or asystolia by the application of 50 Hz alternating current (ac) to electrically driven isolated left atria and right papillary muscles of the guinea pig. An increase in driving frequency from 1 to 3 Hz effected a significant reduction of the threshold of ac-arrhythmia in guinea pig papillary muscle, but no change in atria. A decrease in temperature from 31 °C to 25 °C and an increase in Ca^{2+} from 1.25 to 5 mmol/l elevated the threshold for ac-arrhythmia and -asystolia. Fast sodium channel inhibitors increased threshold of ac-arrhythmia in left atria and papillary muscles, whereas the slow channel inhibitor verapamil was ineffective in concentrations up to 6 µmol/l.

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A.5.0.10
Action potential and refractory period in isolated left ventricular guinea pig papillary muscle

PURPOSE AND RATIONALE

Intracellular action potential in the left ventricular guinea pig papillary muscle is recorded after electrical stimulation. The stimulation frequency is varied in order to determine the refractory period. Resting potential, upstroke velocity, duration of action potential, threshold, refractory period and contractile force can be measured *in vitro*. Compounds which affect the duration of the effective refractory period may have anti-arrhythmic or pro-arrhythmic effects. In addition, the inotropic effect (positive or negative) of the test compound is determined.

PROCEDURE

Guinea pigs of either sex (Marioth strain) weighing 250–300 g are sacrificed by stunning, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed, placed in a container of prewarmed, pre-oxygenated Ringer solution, and the pericardium and the atria are trimmed away. The left ventricle is opened and the two strongest papillary muscles removed. They are fixed between a suction electrode for electrical stimulation and a force transducer for registration of contractions. Ringer solution oxygenated with carbogen (95% O_2/5% CO_2) at a temperature of 36 °C is used.

A standard micro electrode technique is applied to measure the action potential via a glass micro electrode containing 3 M KCl solution, which is inserted intracellularly. The papillary muscle is stimulated with rectangular pulses of 1 V and of 1 ms duration at an interval of 500 ms. The interval between two stimuli is variable in order to determine refractory periods. The intensity of the electrical current is just below the stimulation threshold.

The intracellular action potential is amplified, differentiated for registration of upstroke velocity (dV/dt) (Hugo Sachs micro electrode amplifier), together with the contraction force displayed on an oscilloscope (Gould digital storage oscilloscope OS 4000), and recorded (Gould 2400 recorder).

The effects on fast sodium channels as well as on calcium channels can be studied. The former requires measurement of the normal action potential and the latter the slow action potential obtained at 30 mM K^+. To estimate the relative refractory periods, the second stimuli are set in decremental intervals until contraction ceases. Relative refractory period is defined as the minimum time interval of two stimuli at which each of the stimuli is answered by a contraction. The stimulation threshold is also measured.

After an equilibrium time of 30 min the test compound is added. After 15 and 30 min the following parameters are compared with the predrug values:

- Resting potential mV
- upstroke velocity V/s
- duration of action potential ms
- stimulation threshold V
- refractory period ms
- contraction force mg

The organ bath is flushed thoroughly between two consecutive applications of increasing test drug doses.

EVALUATION

Contractile force [mm] and relative refractory period [ms] are determined before and after drug administration. ED_{25ms}- and ED_{50ms}-values are determined. ED_{25ms} or ED_{50ms} is defined as the concentration of test drug in the organ bath at which the relative refractory period is reduced or prolonged by 25 ms or 50 ms.

Since many anti-arrhythmic agents possess additionally negative inotropic effects, changes in the force of contraction are also determined.

ED_{50} values are calculated from log-probit analyses. Scores are allotted relative to the efficacy of standard compounds (lidocaine, propranolol, quinidine).

The following changes are indicators for anti-arrhythmic activity:

- increase of stimulation threshold
- decrease of upstroke velocity
- prolongation of action potential
- increase of refractory period.

Upstroke velocity and duration of action potential are used for **classification purposes**.

MODIFICATIONS OF THE METHOD

Tande et al. (1990) studied the electromechanical effects of a class III anti-arrhythmic drug on guinea pig and rat papillary muscles and atria using conventional microelectrode technique.

Shirayama et al. (1991) studied with a similar technique the electrophysiological effects of sodium channel blockers in isolated guinea pig left atria.

Dawes (1946) described a method of examining substances acting on the refractory period of cardiac muscle using isolated rabbit auricles.

The same method was recommended as first step of a screening program for quinidine-like activity by Schallek (1956).

Wellens et al. (1971) studied the decrease of maximum driving frequency of isolated guinea pig auricles after antiarrhythmic drugs and beta-blockers.

Salako et al. (1976) recorded electropotentials along the conducting system after stimulation of the proximal part of the His bundle in rabbits.

Brown (1989), Wu et al. (1989), Gwilt et al. (1991a,b) measured *in vitro* transmembrane action potential in Purkinje fibers and endocardial ventricular muscles from dogs.

Voltage clamp techniques in isolated cardiac myocytes from guinea pigs have been used by Wettwer et al. (1991).

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A.6
Methods to induce cardiac hypertrophy and insufficiency

A.6.0.1
Aortic banding in rats

PURPOSE AND RATIONALE

Blood flow restriction of the aorta in rats induces not only hypertension but also cardiac hypertrophy within several weeks. Angiotensin converting enzyme inhibitors, even at subantihypertensive doses, but not other antihypertensive drugs, inhibit cardiac hypertrophy (Linz et al. 1991; Schölkens et al. 1991; Gohlke et al. 1992; Linz 1992a; Linz et al. 1996; Ogawa et al. 1998).

PROCEDURE

Male Sprague Dawley rats weighing 270–280 g are fasted 12 h before surgery. Anesthesia is induced by i.p. injection of 200 mg/kg hexobarbital. The abdomen is shaved, moistened with a disinfectant and opened by a cut parallel to the linea alba. The intestine is moistened with saline and placed in a plastic cover to prevent desiccation. The aorta is prepared free from connective tissue above the left renal artery and underlaid with a silk thread. Then, a cannula no. 1 (0.9×40 mm) is placed longitudinally to the aorta and both aorta and cannula are tied. The cannula is removed, leaving the aortic lumen determined by the diameter of the cannula. The intestine is placed back into the abdominal cavity with the application of 5.0 mg rolitetracycline (Reverin®). In sham-operated controls no banding is performed. The skin is closed by clipping.

The animals are treated once daily over a period of 6 weeks with doses of the ACE-inhibitor or other antihypertensive drugs found previously effective to lower blood pressure in rats. At the end of the experiment blood pressure is measured under hexobarbital anesthesia (200 mg/kg i.p.) via indwelling catheters in the left carotid artery. Blood pressure measurement in conscious rats with the convential tail-cuff method is not possible due to the large pressure difference across the ligature. Therefore, only one measurement at the end of the study is possible. The hearts are removed, rinsed in saline until free of blood and gently blotted to dryness. Total cardiac mass is determined by weighing on an electronic balance to the nearest 0.1 mg. The atria and all adjacent tissues are trimmed off and the weight of the left ventricle including the septum as well as the remaining cardiac tissue representing the right ventricle are determined separately. Weights are calculated per 100 g body weight.

EVALUATION

Total cardiac mass, weight of left and right ventricle of treated rats are compared with operated controls and sham-operated controls.

MODIFICATIONS OF THE METHOD

Uetmasu et al. (1989) described a simple method for producing graded aortic insufficiencies in rats and subsequent development of cardiac hypertrophy. Selective perforation of the right cup of the aortic valve or in combination with that of the left valve cup was performed using a plastic rod inserted from the right common carotid artery. Hypertrophy of the heart, but no hypertension or cardiac insufficiency, was observed.

Similar methods were used by Yamazaki et al. (1989) to study the alterations of cardiac adrenoceptors and calcium channels subsequent to aortic insufficiency, by Umemura et al. (1992) to study baroreflex and β-adrenoceptor function and by Ishiye et al. (1995) to study the effects of an angiotensin II antagonist on the development of cardiac hypertrophy due to volume overload.

Hyperplastic growth response of vascular smooth muscle cells in the thoracic aorta was found following induction of acute hypertension in rats by aortic coarctation by Owens and Reidy (1985).

Prevention of cardiac hypertrophy after aortic banding by ACE inhibitors probably mediated by bradykinin could be shown (Linz et al. 1989, 1992a,b, 1993, 1994; Linz and Schölkens 1992; Schölkens et al. 1991).

Weinberg et al. (1997) studied the effect of angiotensin AT_1 receptor inhibition on hypertrophic remodelling and ACE expression in rats with pressure-overload hypertrophy due to ascending aortic stenosis.

A simple and rapid method of developing high output heart failure and cardiac hypertrophy in rats by producing **aortacaval shunts** was described by Garcia and Diebold (1990). Rats weighing 180–200 g were anesthetized with 30 mg/kg i.p. pentobarbitone. The vena cava and the abdominal aorta were exposed by opening the abdominal cavity via a midline incision. The aorta was punctured at the union of the segment two thirds caudal to the renal artery and one third cephalic to the aortic bifurcation with a 18 gauge disposable needle. The needle was advanced into the aorta, perforating its adjacent wall and penetrating in the vena cava. A bulldog vascular clamp was placed across the aorta caudal to the left renal artery. Once the aorta was clamped, the needle was fully withdrawn and a drop of cyanoacrylate glue was used to seal the aorta punctured point. The clamp was removed 30 s later. The patency of the shunt was verified visually by swelling vena cava and the mixing of arterial and venous blood. The peritoneal cavity was closed with silk thread stitches and the skin with metallic clips. Rats with aorta-caval shunts developed cardiac hypertrophy with significantly higher absolute and relative heart weights.

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A.6.0.2 Cardiac hypertrophy in mice

PURPOSE AND RATIONALE

Rockman et al. (1991, 1993) developed a model of ventricular hypertrophy in the intact mouse by use of microsurgical techniques.

PROCEDURE

Eight week old adult mice weighing 18–22 g are anesthetized by intraperitoneal injection of a mixture of 100 mg/kg ketamine, 5 mg/kg xylazine, and 2.5 mg/kg morphine. Animals are placed under a dissecting microscope in the supine position and a midline cervical incision is made to expose the trachea and carotid arteries. After endotracheal intubation, the cannula is connected to a volume cycled rodent ventilator on supplemental oxygen with a tidal volume of 0.2 ml and a respiratory rate of 110 per min. Both left and right carotid arteries are cannulated with flame stretched PE50 tubing. Catheters are connected to modified P50 Statham transducers.

The chest cavity is entered in the second intercostal space at the left upper sternal border through a small incision and the thymus is gently deflected out of the field of view to expose the aortic arch. After the transverse aorta is isolated between the carotid arteries, it is constricted by a 7.0 nylon suture ligature against a 27-gauge needle, the latter being promptly removed to yield a constriction of 0.4 mm diameter and provide a reproducible transverse aortic constriction of 65–75%.

The hemodynamic effects of acute and chronic constriction are followed by monitoring the pressure gradient between the two carotid arteries in anesthetized animals. Systolic and mean arterial pressure at baseline, during total occlusion when the ligature is tied, and early (15 min) and late (7 days) after transverse aortic constriction are recorded. The increase in systolic pressure provides an adequate mechanical stimulus for the development of cardial hypertrophy.

To confirm myocardial hypertrophy, both sham-operated and aortic-constricted hearts are examined 7 days after operation. Hearts examined for *cell size* are perfused with 4% paraformaldehyde/1% glutaraldehyde through the apex, immersed in osmium tetroxide, dehydrated in graded alcohols, and embedded in araldite. Tissue blocks are sectioned at a thickness of 1 µm, mounted on slides, and stained with toluidine blue. Cell areas are measured by manually tracing the cell outline on an imaging system connected to a computer.

At the end of the experiment, mice were sacrificed in anesthesia, heart excised and weighed, the atria and ventricles separately frozen in liquid nitrogen for Northern blot analysis. Total RNA is extracted by a single step extraction with guanidinium thiocyanate. The RNA is size fractionated by agarose gel electrophoresis, transferred to nylon membranes by vacuum blotting, and hybridized with the appropriate complementary DNA probes labeled with ^{32}P by random priming to a specific activity of $0.95–1.2 \times 10^6$ cpm/ng.

EVALUATION

Variables measured are expressed as mean ±SD. Statistical significance of differences between sham-operated and thoracic aortic-constricted animals is assessed by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Chien (1995) described cardiac muscle diseases in genetically engineered mice:

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A.6.0.3 Cardiac insufficiency in guinea pigs

PURPOSE AND RATIONALE

Congestive heart failure in man is characterized by cardiac hypertrophy, peripheral edema, lung and liver congestion, dyspnea, hydrothorax and ascites. Effective treatment is achieved by cardiac glycosides. Based on techniques reported by Selye et al. (1960) a method was developed to induce congestive heart failure in guinea pigs with symptoms very close to human pathology (Vogel and Marx 1964; Vogel et al. 1965).

PROCEDURE

Male guinea pigs weighing 250–400 g are used. The fur at the ventral thorax is shaved and the skin disinfected. The animal is anesthetized with ether. The skin is cut with scissors on the left side at a length of 4 cm. The left musculus pectoralis is cut at the costal insertion and elevated. The forth intercostal space is opened with two blunted forceps. The heart is pressed against the opening with the left hand. The pericard is opened with a fine forceps and pulled back to the basis of the heart. The beating heart is extruded from the thorax wound by pressure with the left hand on the right thorax wall. A ring-shaped clamp covered with a thin rubber tube is placed around the basis of the heart keeping the heart outside of the thorax without closing off the blood circulation. A thread soaked with diluted disinfectant solution is placed as a loop around the apex of the heart and tightened so that the apical third of both ventricles is tied off. The degree of tightening of the loop is essential. Complete interruption of blood supply to the apical third resulting in necrosis has to be avoided as well as the loop's slipping off. Technical skill is necessary to place the loop around the beating heart into the correct position. After removal of the clamp the heart is placed back, the incision between the forth and fifth costal rib closed and the musculus pectoralis placed over the wound. Intrathoracal air forming a pneumothorax is removed by pressure on both sides of the thorax. After application of an antibiotic emulsion the skin wound is closed. The surgical procedure has to be finished within a short period of time.

The animals develop symptoms of severe congestive heart failure with a death rate of 80% within 14 days. Lung weight and relative heart weight are significantly increased. Exudate in the thorax cavity and ascites amount between 3.5 and 7.5 ml with extreme values of 17.5 ml. Lung edema and liver congestion are found histologically. Peripheral edema and preterminal dyspnea and tachypnea are observed. When treated with various doses (0.1 to 100 µg/kg) of cardiac glycosides s.c. or i.m. over a period of 14 days the symptoms of cardiac insufficiency, e.g., volumes of transudate as well as death rate, are dose-dependent diminished.

EVALUATION

From survival rate, ED_{50} values of cardiac glycosides can be calculated which are in the same dosage range as therapeutic doses in man.

CRITICAL ASSESSMENT OF THE METHOD

The experimental model in guinea pigs reflects very closely the symptoms of cardiac insufficiency in man, e.g., lung congestion, hydrothorax, liver congestion, ascites, peripheral edema and cardiac hypertrophy. The therapeutic potency of cardiac glycosides can be evaluated with this method. Additional factors being known to enhance the symptoms of congestive heart failure in man, like salt load and diphtheria toxin, further increase mortality and hydropic symptoms. The method can be used for special purposes, however, it needs considerable training and technical skill.

MODIFICATIONS OF THE METHOD

Pfeffer et al. (1987) induced myocardial infarction in **rats** by ligation of the left coronary artery and found hemodynamic benefits and prolonged survival with long-term captopril therapy.

Acute ischemic left ventricular failure can be induced in anesthetized **dogs** by repeated injections of plastic microspheres into the left coronary artery (see A.3.2.4).

Kinney et al. (1991) published a method to induce acute, reversible tricuspid insufficiency in anesthetized dogs. A wire spiral is advanced through the atrioventricular canal from the right atrium. The spiral causes regurgitation by preventing complete apposition of the valve leaflets while permitting retrograde flow to occur through the spiral lumen. The degree of regurgitation can be controlled by the use of spirals of different size. Creation of tricuspid insufficiency was demonstrated by onset of right atrial pressure V waves, a ballooning of the right atrium during ventricular systole, palpation of an atrial thrill, or by color Doppler echocardiography. The model is reversible and allows repeated trials of various grades of regurgitation.

Huang et al. (1997) created congestive heart failure in **sheep** by selective sequential intracoronary injection of 90 µm microspheres under 1.5% isofluorane injection.

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A.6.0.4 Cardiomyopathic Syrian hamster

PURPOSE AND RATIONALE

Cardiomyopathy in Syrian hamsters has been described by Bajusz et al. (1966), Bajusz and Lossnitzer (1968), Bajusz (1969), Bajusz et al. (1969a,b), Homburger and Bajusz (1970), Gertz (1972). The disease originates from an autosomal, recessively transmissible disorder which leads to degenerative lesions in all striated muscles and in particular in the myocardium. Histopathological changes consist of myocytolytic necrosis followed by fibrosis and calcification. The evolution of the cardiomyopathic disease can be characterized by five distinct phases: A prenecrotic stage, in which no pathology is evident, a time of active myocytolysis and cellular necrosis, a phase of fibrosis and calcium deposition, an overlapping period of reactive hypertrophy of the remaining viable myocytes, and a final stage of depressed myocardial performance and failure.

PROCEDURE

The model of cardiomyopathy in Syrian hamsters has been used by several authors. One has to note, that several strains of cardiomyopathic hamsters have been used: strain Bio 53:58 by Capasso et al. (1989, 1990) and by Chemla et al. (1992, 1993), strain BIO 14.6 by Tapp et al. (1989) and by Sen et al. (1990), strain CHF 146 CM by van Meel et al. (1989) and by Haleen et al. (1991), strain BIO82.62 by ver Donck et al. (1991), strain J-2-N by Kato et al. (1992), strain CHF 147 by Desjardins et al. (1989), Hanton et al. (1993).

Various experimental protocols have been described. Most authors use survival rate and heart weight as end point (e.g., van Meel et al. 1989; ver Donck et al. 1991; Hanton et al. 1993). Generally, the experiments are started with animals at an age of 120 to 200 days.

Capasso et al. (1989, 1990) studied the mechanical and electrical properties of cardiomyopathic hearts of Syrian hamsters using isolated left ventricular posterior papillary muscles.

Tapp et al. (1989) tested stress-induced mortality in cardiomyopathic hamsters by five consecutive daily 2-h periods supine immobilizations at 4 °C.

Sen et al. (1990) tested the inotropic and calcium kinetic effects of calcium channel agonists and antagonists in primary cultures of isolated cardiac myocytes.

Haleen et al. (1991) tested the effects of an angiotensin converting enzyme inhibitor not only on survival, but also on left ventricular failure in the isolated Langendorff heart by measurement of left ventricular end-diastolic pressure, dP/dt_{max} and mean coronary flow.

Dixon et al. (1997) tested the effect of an AT_1 receptor antagonist on cardiac collagen remodelling in the cardiomyopathic Syrian hamster.

In addition to the effects on left ventricular papillary muscles strips, Chemla et al. (1992) tested the effects on diaphragm contractility in the cardiomyopathic Syrian hamster.

CRITICAL ASSESSMENT OF THE METHOD

Positive effects of various drugs have been found in the cardiomyopathic hamster, such as cardiac glycosides, inotropic compounds, beta-blockers, calcium antagonists, and ACE-inhibitors. The specificity of the effects has to be challenged.

MODIFICATIONS OF THE METHOD

The **tight-skin (TSK) mouse** is a genetic model of pulmonary emphysema connected with right ventricular hypertrophy (Martorana et al. 1990; Gardi et al. 1994).

Valentine et al. (1988) and Devaux et al. (1993) described **X-linked muscular dystrophy in dogs** with cardiac insufficiency similar to Duchenne muscular dystrophy in men and recommended this as an animal model for cardiac insufficiency.

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A.6.0.5 Hypertrophy of cultured cardiac cells

PURPOSE AND RATIONALE

Kojima et al. (1994), Komuro et al. (1990, 1991, 1993), Yamazaki et al. (1993, 1994, 1996) described a method to induce hypertrophy of cardiomyocytes by mechanical stress *in vitro*.

PROCEDURE

Primary cultures from cardiomyocytes are prepared from ventricles of 1-day-old neonatal Wistar Kyoto rats. According to the method of Simpson and Savion (1982), the cultures are treated for 3 days with 0.1 mM bromodeoxyuridine to suppress proliferation of nonmyocardial cells. Elastic culture dishes ($2 \times 4 \times 1$ cm) are made by vulcanizing liquid silicone rubber consisting of methylvinyl polysiloxane and dimethyl hydrogen silicone resin using platinum as a catalyst. The bottom of the disc is 1-mm thick, and it is highly transparent because of no inorganic filler in either component. Cells are plated in a field density of 1×10^5 cells/cm^2 in culture medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Mechanical

stress on cardiac cells is applied by gently pulling and hanging the dish on pegs. A 10% change in length of the dish results in an almost identical change in the length of the cell along a single axis (Kimuro et al. 1990). Cardiocytes are stretched by 5%, 10%, or 20%. Drugs, e.g., an angiotensin-II receptor antagonist, are added 30 min before stretch.

For protein analysis, the silicone dishes are stretched for 24 h after 2 days of serum starvation and [^{3}H]phenylalanine (1 μCi/ml) is added for 60 min. At the end of each stress, the cells are rapidly rinsed four times with ice-cold phosphate-buffered saline and incubated for 20 min on ice with 1 ml of 5% trichloroacetic acid. The total trichloroacetic acid-insoluble radioactivity in each dish is determined by liquid scintillation counting.

For determination of mitogen-activated protein kinase, cardiomyocytes are lysed on ice and centrifuged. Aliquots of the supernatants of myocyte extracts are incubated in kinase buffer (25 mM/l Tris-HCl, pH 7.4, 10 mM/l $MgCl_2$, 1 mM/l dithiothreitol, 40 μM/l APT, 2 μCi [γ-^{32}P]ATP, 2 μM/l protein kinase inhibitor peptide, and 0.5 mM/l EGTA) and substrates (25 μg myelin basic protein). The reaction is stopped by adding stopping solution containing 0.6% HCl, 1 mM/l ATP, and 1% bovine serum albumin. Aliquots of the supernatant are spotted on P81 paper (Whatman), washed in 0.5% phosphoric acid, dried and counted.

For determination of *c-fos mRNA*, Northern blot analysis is performed.

EVALUATION

Values are expressed as mean ±SEM. Comparisons between groups are made by one-way ANOVA followed by Dunnett's modified *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The interesting approach to induce hypertrophy of cardiac cells *in vitro* has been used predominantly by one research group. Confirmation by other research groups including modifications of the mechanical procedures seems to be necessary.

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A.7 Positive inotropic activity (cardiac glycosides)

A.7.0.1 General considerations

Biological standardization of cardiac glycosides was necessary as long as the drugs used in therapy were plant extracts or mixtures of various glycosides. They were standardized in units of an international standard. Some of the pharmacological methods used for these purposes and adopted by many pharmacopoeias have nowadays *historical interest* only. This holds true for the frog method and the pigeon method (Burn et al. 1950).

Particularly, the **frog method** was used for standardization. The method adopted by the U.S Pharmacopoeia X was the 1 h test. Healthy frogs (*Rana pipiens*) weighing 20–30 g were selected from the cold storage room. One hour before assay, their weight was recorded and they were placed in wire cages with a water depth of 1 cm. The doses of digitalis were calculated so that they approximated 0.015 ml/g body weight. Injections were made into the ventral lymph sac. One hour later, the animals were pithed and the heart removed and examined. Systolic arrest of the ventricle and widely dilated atrium indicated the typical result. Calculations of activity in terms of International Units were made from the percentage of dead animals in the test group versus those in the group receiving the international standard.

The **pigeon method** introduced by Hanzlik (1929) and adopted by USP XVII depends on the observation that intravenously injected cardiac glycosides have an emetic action in pigeons. In the original test, adult pigeons weighing 300–400 g are injected with a solution of the cardiac

glycoside into a suitable wing vein in the axillary region. Vomiting occurring within 15 min is regarded as positive result. Two doses of test solution and standard are injected and percentage of vomiting pigeons registered. This 4 point assay allows calculation of ED_{50} values and of the potency ratio compared with the standard.

Modifications of other methods, such as the **cat method** introduced by Hatcher and Brody (1910) and described in detail by Lind van Wijngarden (1926), the **guinea pig method** described by Knaffl-Lenz (1926) and the **isolated cat papillary muscle** method introduced by Catell and Gold (1938) still being used for evaluation of synthetic cardioglycosides and other positive inotropic compounds are referenced in detail below.

Surveys on the evaluation of cardiac glycosides have been given by Bahrmann and Greef (1981), for the use of the isolated papillary muscle by Reiter (1981) and for other isolated heart preparations by Greef and Hafner (1981). Moreover, the influence on Na^+/K^+-ATPase, an *in vitro* model specific for cardiac glycosides (Gundert-Remy and Weber 1981), is described.

The mechanisms of action have been reviewed by Scholz (1984) and Grupp (1987).

Analogous to antiarrhythmic agents, Feldmann (1993) proposed a classification system that categorizes inotropic agents according to their supposed mode of action:

Class I: Inotropic agents that increase intracellular cyloAMP, including β-adrenergic agonists and phosphodiesterase inhibitors,
Class II: Inotropic agents affecting sarcolemmal ion pumps and channels, in particular cardiac glycosides inhibiting Na^+/K^+-ATPase,
Class III: Agents that modulate intracellular calcium mechanisms (no therapeutic inotropic agents in this kind yet available),
Class IV: Inotropic agents having multiple mechanisms of action

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A.7.1 *In vitro* tests

A.7.1.1 Ouabain binding

PURPOSE AND RATIONALE

Cardiac glycosides can be characterized by their binding kinetics (association process, equilibrium binding, and dissociation process) on the ouabain receptor.

PROCEDURE

Heart sarcolemma preparations are obtained from rat or dog heart. From a canine heart or from rat hearts submitted to coronary perfusion myocytes are isolated by collagenase digestion. The isolated membrane fractions consist mainly of myocyte sarcolemma. [^{3}H]ouabain with a specific radioactivity of about 20 Ci/mmol is incubated with ligands to be tested in 10 ml of binding medium consisting of 1 mM $MgCl_2$, 1 mM inorganic phosphate, and 50 mM Tris-HCl, pH 7.4 at 37 °C for 10 min.

Association process: After temperature equilibration in the presence of either 10 or 100 nM [^{3}H]ouabain, 200 µg of membrane preparation are added to initiate the reaction. At various times, 4.5 ml are removed and rapidly filtered.

Equilibrium binding: At the end of the temperature equilibration carried out in the presence of increasing concentrations of [^{3}H]ouabain ranging from 10 nM to 3 µM, 40 µg of membranes are added. After 30 min, duplicate aliquots of 4.5 ml are removed and filtered.

Dissociation process: Once equilibrium has been achieved under the experimental conditions used to study association, 10 ml of prewarmed Mg^{2+} plus P_i Tris-HCl solution supplemented with 0.2 mM unlabeled ouabain are added to initiate dissociation of [^{3}H]ouabain. At various times, aliquots of 0.9 ml are removed and rapidly filtered.

All aliquots are filtered under vacuum on HAWP Millipore filters (0.45 µm) and rinsed three times with 4 ml of ice-cold buffer. The radioactivity bound to the filters and the specific binding measurements are determined.

EVALUATION

Kinetic parameters for the association and the dissociation process are calculated. The results of equilibrium binding are analyzed by Scatchard plots.

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A.7.1.2 Influence on Na^+/K^+ ATPase

PURPOSE AND RATIONALE

The enzyme Na^+/K^+ ATPase is the transport system for Na^+ and K^+ in the cell membranes. The membrane bound enzyme couples ATP hydrolysis to the translocation of Na^+ and K^+ ions across the plasma membrane through a series of conformational transitions between the E_1 and E_2 states of the enzyme. The enzyme is a heterodimer consisting of a catalytic subunit (110 kDa) associated with a glycosylated β subunit (55 kDa). Three alpha (α_1, α_2, α_3) subunits have been identified by cDNA cloning. In the heart, enzyme Na^+/K^+ ATPase is the target of the positive inotropic glycosides. Therefore, it is of interest for the characterization of positive inotropic compounds. The test is based on the determination of phosphate generated from ATP under special conditions. Inhibition of bovine cerebral Na^+/K^+ ATPase prepared according to Schoner et al. (1967) is measured after addition of various concentrations of the test compound compared with those of the standard (Erdmann et al. 1980).

PROCEDURE

Solutions

1.00 ml	133 mM imidazole pH 7.3
0.04 ml	160 mM $MgCl_2$
0.02 ml	DPNH (10 mg/ml)
0.04 ml	310 mM NH_4Cl
0.04 ml	100 mM ATP
0.02 ml	40 mM phospho-enolpyruvate
0.05 ml	pyruvate-kinase (1 mg/ml = 150 U/ml)
0.04 ml	lactate-dehydrogenase (0.5 mg/ml = 180 U/ml)
0.20 ml	1 M NaCl
0.01–0.02 ml	bovine cerebral ATPase (depending on activity of the enzyme) up to 2.0 ml distilled water

Test

The enzyme activity is started by addition of the ATP solution at 37 °C. After 4 min the inhibitor (various concentrations of the cardiac glycoside) is added. Na^+/K^+ ATPase activity is measured by a coupled optical assay. The reaction is continuously recorded and corrected for Mg^{2+}-activated ATPase by inhibition of Na^+/K^+ ATPase with 10^{-3} M ouabain.

EVALUATION

Inhibition of ATPase is measured after addition of various concentrations of the test compound. Dose-response curves are established and compared with the standard (k-strophanthin). Potency ratios can be calculated.

MODIFICATIONS OF THE METHOD

Brooker and Jelliffe (1972) and Marcus et al. (1975) described an *in vitro* assay based on displacement of radiolabeled ouabain bound to ATPase by various glycosides. Another method is based on the inhibition

of rubidium uptake into erythrocytes (Lowenstein 1965; Belz 1981).

Erdmann et al. (1980) prepared (Na^++K^+)-ATPase-containing cardiac cell membranes from rat hearts.

Maixent et al. (1987, 1991) described two Na,K-ATPase isoenzymes in canine cardiac myocytes as the molecular basis of inotropic and toxic effects of digitalis.

The effect of ouabain on Na^+/K^+ATPase activity in cells of the human rhabdomyosarcoma cell line TE671 was studied by Miller et al. (1993) with a special equipment, the microphysiometer (McConnell et al. 1992).

CRITICAL ASSESSMENT OF THE METHOD

The *in vitro* methods being used for determinations of plasma levels of glycosides (Maixent et al. 1995) have been largely substituted by radio-immunoassays specific for individual glycosides. Nevertheless, the inhibition of Na^+/K^+ ATPase can be used as an indicator of activity of new semisynthetic cardiac glycosides.

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A.7.2
Tests in isolated tissues and organs

A.7.2.1
Isolated cat papillary muscle

PURPOSE AND RATIONALE

Isolated cardiac tissue has been chosen to study the decrease of performance after prolonged electrical stimulation and during restoration of force under the influence of cardiac glycosides. Cattell and Gold (1938) described a method using cat papillary muscle.

PROCEDURE

Cats of either sex weighing 2.5–3 kg are used. The animal is anesthetized with ether and the thorax is opened rapidly. The heart is removed and a papillary muscle from the right ventricle is isolated and fixed in an organ bath containing oxygenated Ringer's solution at 36 °C. One end of the muscle is tied to an tissue holder and the other one to a strain gauge. The muscle is stimulated electrically with 4–6 V, 2 ms duration and a rate of 30/min. The contractions are recorded on a polygraph. After 1 h, the muscle begins to fail and the force of contraction diminishes to a fraction of control. At this point, the cardiac glycoside is added to the bath, restoring the contractile force to levels approaching control. The standard dose is 300 ng/ml ouabain. The potency of natural and semisynthetic glycosides can be determined with this method. Catecholamines, like adrenaline (10 ng/ml) or isoprenaline (10 ng/ml), are active as well.

EVALUATION

The increase of contractile force is calculated as percentage of the predose level. Dose-response curves can be established using various doses.

CRITICAL ASSESSMENT OF THE METHOD

The use of isolated papillary muscle strips can be recommended for evaluation of inotropic compounds of various chemical classes.

MODIFICATIONS OF THE METHOD

Instead of cat papillary muscle the isolated left atrium of guinea pigs can be used (see Sect. A.1.2.6). For testing cardiac glycosides, the calcium content in the Ringer solution is reduced to 50%.

Andersom (1983) compared responses of guinea pig paced left atria to various positive inotropic agents at two different calcium concentrations (1.25 and 2.50 mM). Consistently good results were obtained at the lower calcium concentration with isoproterenol, ouabain, amrinone, and 3-isobutyl-1-methylxanthine.

Böhm et al. (1989) studied positive inotropic substances like isoprenaline and milrinone in isolated cardiac preparations from different sources. They used isolated papillary muscles from Wistar-Kyoto rats and from spontaneously hypertensive rats, but also human papillary muscle strips from patients with moderate heart failure (NYHA II–III) and compared the effects with papillary muscle strips from patients with severe heart failure (NYHA IV). They recommended that new positive inotropic agents should be screened in human myocardial tissue from patients with heart failure.

Labow et al. (1991) recommended a human atrial trabecular preparation for evaluation of inotropic substances.

Böhm et al. (1989) tested positive inotropic agents in isolated cardiac preparations from different sources, e.g., human papillary muscle strips from patients with severe heart failure (NYHA IV), human papillary muscle strips from patients with moderate heart failure (NYHA II–III), human atrial trabeculae, isolated papillary muscles from Wistar-Kyoto rats, and isolated papillary muscles from spontaneous hypertensive rats. They suggested that positive inotropic effects should be screened in isolated myocardium from patients with heart failure.

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A.7.2.2 Isolated hamster cardiomyopathic heart

PURPOSE AND RATIONALE

Special strains of Syrian hamsters develop cardiomyopathy. These animals can be used for evaluation of cardiotonic drugs (see Sect. A.6.0.4).

PROCEDURE

Hamsters with cardiomyopathy (Bio 14/6) at the age of 50 weeks are used. Controls are normal Syrian hamsters (FIB hybrids) at the same age. The animals are pretreated with heparin (5 mg/kg i.p.) and 20 min later the heart is prepared according the method of Langendorff and perfused with heart Ringer solution under 75 mm H_2O hydrostatic pressure. The preparation is allowed to equilibrate in the isolated state for 60 min at 32 °C with a diastolic preload of 1.5 g. The force of contractions is recorded isometrically by a strain gauge transducer on a polygraph, e.g., Heliscriptor He 19 recorder (Hellige GmbH, Freiburg, Germany). From these signals, the heart rate is measured by a chronometer. The coronary flow is measured by an electromagnetic flowmeter. Compounds are injected via the aortic cannula into the inflowing heart-Ringer solution.

EVALUATION

The contractile force and the coronary flow in hearts from diseased and normal animals is registered before and after application of the test drugs. Mean values and standard deviation are calculated before and after drug application and statistically compared using Student's *t*-test.

MODIFICATION OF THE METHOD

Jasmin et al. (1979) showed after prolonged *in vivo* administration beneficial effects of a variety of cardiovascular drugs, including verapamil, prenylamine, dibenamine and propranolol.

After chronic administration (4 or 12 weeks subcutaneously), Weishaar et al. (1987) found beneficial effects of the calcium channel blocker diltiazem, but not by the administration of digitalis.

In contrast, in the experiments of Ottenweller et al. (1987) hamsters treated orally with digoxin survived and showed significant amelioration of the pathological syndrome of heart failure.

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A.7.2.3 Potassium loss from the isolated guinea pig heart

PURPOSE AND RATIONALE

Cardiac glycosides induce a net loss of potassium from cardiac tissue due to their inhibition of the Na^+/K^+ ATPase. Therefore, potassium is increased in the effluent of the isolated guinea pig heart. This phenomenon can be used as parameter for the activity of digitalis-like compounds (Lindner and Hajdu 1968).

PROCEDURE

The isolated heart of guinea pigs according to LANGENDORFF is prepared as described in Sect. A.3.1.1. The coronary outflow is measured by counting the drops of the effluent by a photocell. The effluent is collected in a funnel with a thin upwards shaped outlet allowing to withdraw small fluid samples for analysis by a flame photometer. A pump attached to a 4-way valve changes the samples to the flame photometer every 15 s in the following sequence: effluent Tyrode-solution from the heart, distilled water, Tyrode-solution used for perfusion, distilled water. The potassium content of affluent and effluent Tyrode-solution is compared and registered on a Varian®-recorder. The difference is attributed to the potassium outflow from the heart. The dose-response curve is flat in the therapeutic range, much steeper in the toxic range.

EVALUATION

The following parameters are recorded and calculated:

- coronary flow [ml/min]
- contractile force
- potassium loss [mVal/min]

CRITICAL ASSESSMENT OF THE METHOD

A good correlation was found between the measured potassium loss and the positive inotropic effect of cardiac glycosides. The method is suitable for the quick determination of efficacy of digitalis-like substances and facilitates the discrimination from other positive inotropic compounds like adrenaline.

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A.7.3 *In vivo* tests

A.7.3.1 Cardiac toxicity in cats (Hatcher's method)

PURPOSE AND RATIONALE

The purpose of the method, originally introduced by Hatcher and Brody (1910) and described in detail by Lind van Wijingaarden (1926), was to establish "cat units" for cardiac glycoside preparations. Hatcher and Brody defined "the cat unit as the amount of crystalline ouabain which is fatal within about ninety minutes to a kilogram of a cat when the drug in injected slowly and almost continuously into the femoral vein". Time to cardiac arrest after intravenous infusion of a solution with defined concentration of the standard was used as reference and the unknown solution of the test preparation compared with the standard. The method can be used for testing natural and semisynthetic glycosides.

PROCEDURE

Cats of either sex weighing 2–3.5 kg are temporarily anesthetized with ether. Anesthesia is maintained with 70 mg/kg chloralose given intravenously. The animal is fixed on its legs on a heated operating table. Tracheostomy is performed and a tracheal cannula is inserted. ECG is recorded from lead II. Then, intravenous infusion of the test solution is started. The endpoint is cardiac arrest which should be reached within 30–60 min by proper adjustment of the concentration of the infused solution.

MODIFICATIONS OF THE METHOD

Hatcher's original method has been modified by many authors. The method using **guinea pigs**, introduced by Knaffl-Lenz (1926) is in its essentials similar to the cat method.

Guinea pigs weighing 400–600 g are anesthetized with urethane (1.75 g/kg i.m.) The animal is secured on a operating table and the trachea is cannulated. The jugular vein is cannulated for infusion of the test preparation. Cardiac arrest is recorded from ECG lead II.

Dogs and guinea pigs were used by Dörner (1955).

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A.7.3.2 Decay rate and enteral absorption rate of cardiac glycosides

PURPOSE AND RATIONALE

The basic principle of Hatcher's or Knaffl-Lenz's method is suitable to determine decay rates of cardiac glycosides. The decay of efficacy can by due to excretion or metabolic degradation of the glycoside.

PROCEDURE

Beagle dogs of either sex weighing 8–20 kg are anesthetized with 35 mg/kg pentobarbital sodium i.v. The animal is fixed on its legs on a heated operating table. Tracheostomy is performed and a tracheal cannula inserted. The vena femoralis is cannulated for continuous infusion of a defined concentration (μg/kg/min) of the test compound. ECG is recorded from lead II. The signs of first toxic effects, e.g., extra systoles, AV-block) are recorded. At this time, the infusion is terminated and the total dose/kg of the applied glycoside registered. After 4, 8, 12, or 24 h the infusion procedure is repeated. Within this period of time the glycoside administered with the first dose is only partially metabolized or excreted. Therefore, the dose needed for observation of ECG changes during the second infusion will be lower than in the first experiment.

EVALUATION

The dose required in the second experiment for induction of ECG changes is equal to the amount of metabolized or excreted glycoside. This value is expressed as percentage of the amount required in the first experiment and indicates the decay rate of the glycoside. Testing after various time intervals, the decay rate can be visualized graphically and half life times be calculated.

MODIFICATIONS OF THE METHOD

Rhesus monkeys have been used since their response to cardiac glycosides is more similar to that of man than that of dogs (Lindner et al. 1979).

The basic principle of Hatcher's or Knaffl-Lenz's method is also suitable to determine *enteral absorption of cardiac glycosides*. Again, for this purpose dogs

are preferred instead of cats or guinea pigs. The dose to induce cardiac arrest is determined in 3–6 dogs. To other dogs, the same test compound is given intraduodenally at a dose below the intravenous lethal dose. Ninety or 180 min afterwards, the intravenous infusion with the same infusion speed and the same concentration of the test compound as in the previous experiments is started and time until cardiac arrest determined. The higher the duodenal resorption of the compound, the lower the dose of the intravenous infusion will be. For evaluation, the intravenous dose needed in the second experimental series (with enteral predosing) is subtracted from the dose of the first series (without enteral predosing) and indicates the amount of absorbed compound. This value is expressed as percentage of the value of the first series and indicates the absorption rate.

The efficacy and safety of a novel Na^+,K^+-ATPase inhibitor has been tested in dogs with propranolol-induced heart failure by Maixent et al. (1992).

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A.8
Effects on blood supply and on arterial and venous tonus

A.8.1
Models for stroke and multi-infarct cerebral dysfunction

A.8.1.1
Cerebral ischemia by carotid artery occlusion in Mongolian gerbils

PURPOSE AND RATIONALE

The Mongolian gerbil (*Meriones unguiculatus*) is extremely susceptible to carotid occlusion because of the peculiar anatomical occurrence of an incomplete circle of Willis without posterior communicating artery and a frequently rudimentary anterior communicating artery. Clamping of both carotid arteries induces a bilateral temporary brain ischemia (Levine and Sohn 1969; Bosma et al. 1981; Mršulja et al. 1983; Hossmann et al. 1983; Chandler et al. 1985). This pathological animal model allows the simulation of circulatory disturbances in the human brain. The hippocampus is one of the most vulnerable regions of the brain to ischemia and anoxia. The gerbil is known to develop selective neuronal damage in the CA1 sector of the hippocampus following brief periods of forebrain ischemia. This damage differs from conventionally described ischemic neuronal injury because of its slow development (Ito et al. 1975; Kirino 1982; Hossmann et al. 1983). The occlusion time can be varied allowing determination of various parameters, e.g. ischemia induced amnesia (see Sect. F.3.1.8).

PROCEDURE

Male Mongolian gerbils (strain: Hoe GerK jirds) weighing 48–88 g are randomly divided into groups (10–15 animals for each test and control group). Prior to testing, the animals are housed in a climate-controlled environment (21 °C) with food and water available ad libitum. Fifteen minutes before surgery, the gerbils receive the test compound by oral or intraperitoneal administration. The control group is treated with vehicle alone.

The exposure of the common carotid arteries is performed under anesthesia with sodium pentobarbital (32 mg/kg i.p.), chloralhydrate (100 mg/kg i.p.) and atropine sulfate (0.8 mg/kg i.p.). The carotid arteries are isolated from surrounding tissue and a loop of unwaxed dental floss is placed around each artery. A 2 cm length of double lumen catheter is passed from the level of carotid artery through the muscle layers of the dorsal surface of the neck. Each end of dental floss is threaded through a separate lumen, leaving a loose loop around the artery. Two days later, occlusion of each artery is produced by gently pulling the dental floss until the artery is completely occluded between the floss and the center wall of the catheter. Heifitz clips are placed on the floss against the exterior end of the tubing to maintain occlusion. After various intervals (5 to 30 min), the clips are removed and circulation is restored.

Complete bilateral occlusion of the arteries is confirmed by behavioral symptoms, i.e., depression of spontaneous motor activity, shallow and rapid respiration, and ptosis. Care is taken to avoid a drop of body temperature during any stage of the experiment. After experimental manipulations, animals are placed on a heating pad until complete recovery of motor activity.

Subjects are placed in individual observation glasses which are kept at a temperature of 29 °C. They are observed for neurological symptoms (such as circling behavior, jumping and rolling seizures, opisthotonus, tonic convulsions, etc.) for 90 min.

After various intervals, the gerbils are sacrificed and their brains are removed.

EVALUATION

The following parameters are measured two hours after occlusion:

Degree of brain edema: water content (difference in weight of wet and dry brain)

Content of sodium and potassium. The hemispheres are separated and put on pre-weighted watch glasses to determine the wet weight. Then the hemispheres are dried in an open Petri dish for 2 days at a temperature of 95 °C. After cooling off, the dry weight is noted. Sodium and potassium concentrations in the dried brain hemispheres are determined by flame photometry. The Na^+/K^+-ratio is calculated.

For *histological examinations*, the animals are sacrificed at 2 or 4 days after ischemia under ether anesthesia by decapitation. The brains are then removed and frozen in CO_2. Hippocampi are sectioned coronally with a cryostat at –14 °C. The section thickness for Nissl and glial fibrillary acid protein (GFAP) staining is 20 µm and for the histochemical localization of calcium 30 µm.

Nissl staining and its quantitative evaluation. The sections are mounted by thawing on glass slides, Nissl stained, and cover slipped with Permount. In order to standardize the histological procedure and to rule out the possibility that differences in the staining intensity were due to technical inconsistency, slides from control and experimental groups are processed together, stained for 5 min, and differentiated in a series of alcohols for 3 min each. The extent of hippocampal nerve cell damage (as reflected by cell loss and decreased stainability) is assessed by measuring the amount of Nissl-stained material in a predetermined representative region of the CA1 area with the aid of a guided densitometer (Leitz Texture Analysis System). The measuring field is 50×500 µm, fitting to the width of the CA1 soma layer (about 40 µm).

Calcium localization. A modification of technique described by Kashiwa and Atkinson (1963) is used for the cytochemical localization of ionic calcium. The principle of the technique is that calcium complexes with a chelating agent producing an insoluble chromophore.

Stock solutions: Two solutions were used: a) glyoxal-bis-(2-hydroxyanil) (GBHA), 0.4 g/100 ml absolute ethanol (2 ml 0.4% GBHA); and b) NaOH, 5 g/100 ml distilled water (0.3 ml 5% NaOH).

Staining procedure: First, cryostat sections are placed immediately in cold absolute acetone for rapid fixation for 5 min. Next, floating sections are transferred into 96% alcohol for 5 min and then transferred to staining solution for 3–4 min. Sections are then placed in 96% alcohol and mounted on glass slides. Because of quenching, it is necessary to view and photograph immediately.

GFAP (glial fibrillary acid protein) fluorescencemicroscopy. Following slide mounting, cryostat sections are fixed for 15 min in 3.5% formaldehyde solution in 0.01 M phosphate-buffered saline (PBS). The sections are incubated with mouse primary antibody against GFAP (Boehringer, Mannheim, FRG) for 30 min diluted 1:50 PBS. This antibody shows cross reactions also with GFAP from pigs and rats, indicating low species selectivity (Graber and Kreutzberg, 1986). The sections are rinsed in PBS and incubated to tetramethylrhodamine isothiocyanate (TRITC) specific for mouse immunoglobulin G (T-5393 from Sigma) diluted 1:50 in PBS. Control sections are incubated with PBS instead of primary antibody.

Measurements, expressed as extinction units per measuring field, are taken from three slides of each animal and averaged. Statistical analysis is done by Student's *t*-test.

MODIFICATIONS OF THE METHOD

"Sensitive" gerbils can be selected according to the method of Delbarre et al. (1988). In this method, pupil dilatation is obtained with atropine sulfate (1%) 20 min before anesthesia. Ocular fundus is examined with direct ophthalmoscope (Heine) before ligation and 5 min later. Only animals with an absence of retinal blood flow after ligation are considered as positive.

Using ^{31}P nuclear magnetic resonance spectroscopy, Sasaki et al. (1989) studied energy metabolism of the ischemic brain of gerbils *in vivo*.

An unanesthetized-gerbil model of cerebral ischemia was described by Chandler et al. (1985).

Using microdialysis, adenosine and its metabolites were measured directly in the brain of male gerbils by Dux et al. (1990). Two microdialysis probes (CMA/10, Carnegie Medicine, Sweden) were implanted stereotactically in the brain of the animals, one in the left dorsal hippocampus and one in the right striatum. The fibres were fixed to the cranium using dental wax. The dialysate was collected in 5 min intervals, and the concentrations of adenosine, hypoxanthine and inosine were determined by HPLC (Zetterström et al. 1982; Fredholm et al. 1984).

Kindy et al. (1992) measured glial fibrillary acid protein and vimentine on mRNA level by Northern blot analysis and protein content by immunoblot analysis in the gerbil neocortex, striatum and hippocampus after transient ischemia.

McRae et al. (1994) studied the effect of drug treatment on activated microglial antigens in hippocampal sections after ischemia in gerbils with cerebral fluid from patients with Alzheimer's disease and the amyloid precursor protein.

Nurse and Corbett (1996) found neuroprotection in gerbils with global cerebral ischemia after several days of mild, drug-induced hypothermia. The protection by the AMPA-antagonist NBQX may be due to a decrease in body temperature. A protracted period of subnormal temperature during the postischemic period can obscure the interpretation of preclinical studies.

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A.8.1.2 Forebrain ischemia in rats

PURPOSE AND RATIONALE

Smith et al. (1984) described a model of forebrain ischemia in rats induced by transient occlusion of both carotid arteries and exsanguination to a blood pressure of 40 mm Hg. This method has been used extensively (Nuglisch et al. 1990; Oberpichler et al. 1990; Rischke and Krieglstein 1990; Krieglstein and Peruche 1991; Nuglisch et al. 1991; Prehn et al. 1991; Rischke and Krieglstein 1991; Seif el Nasr et al. 1992; Peruche et al. 1995).

PROCEDURE

Male Wistar rats weighing 250–300 g are anesthetized with 3.5% halothane and then connected to a Starling type respirator delivering 0.8% halothane and 30% O_2 in N_2O. The jugular vein and the tail artery are catheterized for withdrawal of blood and for monitoring blood pressure. Anticoagulation is achieved by intravenous heparin (200 IU/kg) application. Blood gases, blood pH, blood pressure, and blood glucose are measured 5 min prior to ischemia and 10 min after ischemia.

Halothane, but not N_2O, is discontinued and the rats are allowed to recover for 30 min. During this period, muscle paralysis is maintained with 5 mg/kg suxamethonium chloride, repeated every 15 min. After injection of trimethaphan camphor sulfonate (5 mg/kg),

forebrain ischemia is induced by clamping of both carotid arteries and exsanguination to a blood pressure of 40 mm Hg. To prevent decay of intra-ischemic brain temperature, the environmental temperature is adjusted to 30 °C by means of a infrared heating lamp. After 10 min of ischemia, the carotid clamps are removed and blood pressure is restored by re-infusing the shed blood. To minimize systemic acidosis, the rats receive intravenously 50 mg/kg $NaHCO_3$. The animals are removed from the respirator when they regain spontaneous respiration.

EVALUATION

Various parameters are used to evaluate the consequences of transient forebrain ischemia and the effectiveness of drug treatment:

- Local cerebral blood flow determination with the [^{14}C]iodoantipyrine method (Sakurada et al. 1978),
- Histological assessment of ischemic cell damage in the hippocampus on day 7 after ischemia (Seif el Nasr et al. 1990; Nuglisch et al. 1990),
- Local cerebral glucose utilization using the [^{14}C]deoxyglucose method described by Sokoloff et al. (1977),
- Quantitative analysis of the electrocorticogram (Peruche et al. 1995).

MODIFICATIONS OF THE METHOD

Kochhar et al. (1988) used two focal cerebral ischemia models in **rabbits**: a multiple cerebral embolic model by injection of microspheres into the internal carotid circulation and a spinal chord ischemia model by occluding the aorta for predetermined periods.

Gilboe et al. (1965) described the isolation and mechanical maintenance of the **dog** brain.

Andjus et al. (1967) and Krieglstein et al. (1972) described the preparation of the isolated perfused rat brain for studying effects on cerebral metabolism.

A cerebral ischemia model with conscious **mice** was described by Himori et al. (1990).

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A.8.1.3 Hypoxia tolerance test in rats

PURPOSE AND RATIONALE

The electrical activity of the brain is dependent on a continuous energy supply. Hypoxia is induced in test animals by inhalation of nitrogen. Marked hypoxia depresses cerebral metabolism resulting in an electri-

cal failure of the brain. The procedure is used to investigate the ability of test compounds to antagonize the hypoxia-induced electrical failure of the brain by measuring the hypoxia-tolerance and the EEG recovery.

PROCEDURE

Male Sprague Dawley rats or stroke-prone rats weighing 250–300 g are used. They are anesthetized with hexobarbital sodium (100–120 mg/kg, i.p) and surgically implanted with 2 epidural EEG-electrodes and a reference electrode to the parietal frontal cortex. After a minimum of a one week recovery period, testing can be started. The rats receive the test compound by intravenous or intraperitoneal administration. The control group is treated with vehicle alone. Thirty to 60 min after i.p. administration (immediately after i.v. administration) the animals are anaesthetized by hexobarbital sodium at 100–120 mg/kg, i.p. When the stage of surgical anesthesia is reached, the animals are placed in a hypoxia chamber. EEG and ECG are recorded using a Hellige recorder.

Hypoxia is induced by inhalation of nitrogen (1 200 l/h). On reaching an isoelectric EEG, the nitrogen inhalation is terminated and the animals are allowed to breath room air. The recording is continued until EEG potentials can again be registered.

EVALUATION

The following parameters are measured in the test and control groups

- Hypoxia tolerance (HT): The time from the start of nitrogen inhalation until the onset of isoelectric EEG.
- Latency of EEG recovery: The time from the end of nitrogen inhalation until the onset of EEG potentials.
- The values of test and control groups are compared.

The percent change is calculated.
Statistics: Student's *t*-test by unpaired comparison.

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A.8.1.4 Middle cerebral artery occlusion in rats

PURPOSE AND RATIONALE

The permanent middle cerebral artery (MCA) occlusion technique in rats has been widely employed to evaluate various kinds of neuroprotective agents in cerebral ischemia (Ginsberg and Busto 1989). Focal cerebral infarction is achieved using a modification of the MCA occlusion model described by Tamura et al. (1981) and Pak et al. (1992).

PROCEDURE

Adult male Sprague-Dawley rats weighing 300–400 g are used in the experiments. The animals are anesthetized with a nitrous oxide-oxygen mixture (70:30%) containing 1.0 to 1.2% halothane. A mask is put on the nose and self-respiration is maintained during surgery, which takes around 20 to 30 min. The right femoral artery and vein are catheterized for monitoring blood pressure, blood sampling and administration of the drug. Left MCA occlusion is performed via a subtemporal approach without removal of zygomatic arch or temporal muscle. Under high magnification of a surgical microscope, the left MCA is coagulated with a microbipolar coagulator from the olfactory tract to the most proximal portion of the MCA through a cranial window, about 3 to 4 mm in diameter, and cut afterward. Anesthesia is stopped just after MCA occlusion. The arterial catheter is removed soon after MCA occlusion, but the venous catheter is maintained for constant infusion of the drug by a swivel system (Harvard Apparatus, UK.). The animals are maintained normothermic (37 °C) by a homeothermic system (Homeothermic Blanket System, Harvard Apparatus, UK.). During the surgery, mean arterial blood pressure (MABP) is recorded continuously (Model 7E polygraph, Grass, USA). Arterial gases and pH (178 pH/blood gas analyzer, Corning, USA) as well as hematocrit and blood glucose are measured twice, once about 15 min before MCA occlusion and the other just following MCA occlusion. Bilateral temporal muscle temperature (Therm 2250-1, Ahlborn Mess- und Regelungstechnik, Germany) is monitored during the surgery and several minutes before sacrifice.

Two, six, twelve and twenty-four hours after discontinuing anesthesia, each rat's level of consciousness and motor activity are evaluated using a grading scale of 0 to 3 (0: normal activity; 1: spontaneous activity+/–; 2: not arousable by tactile stimulation; 3: not arousable by painful stimulation, no spontaneous activity). Immediately before sacrifice, neurological status is examined using a grading scale of 0 to 3 (0: no observable deficit; 1: forelimb flexion; 2: decreased resistance to lateral push without circling; 3: the same status as grade 2, with circling). Four groups of rats are studied: vehicle-treated controls, and drug-treated animals at 3 different doses.

All the rats are re-anesthetized 24 h after MCA occlusion and sacrificed by intravenous injection of KCl. Immediately after sacrifice the brain is removed and frozen at –10 °C for 10 min. The forebrain is cut into eight coronal slices by a rat brain slicer which are proc-

essed with the tetrazolium chloride (TTC) emulsion technique. Areas of brain not stained by TTC are drawn on a diagram at 8 preselected coronal levels of forebrain without knowledge of the experimental treatment. The areas of ischemic damage in the cerebral hemisphere, cerebral cortex, and caudate nucleus, drawn in the diagram are measured with a planimeter (KP-21, Koizumi, Japan) and integrated to determine the volumes.

EVALUATION

Significance of the differences between the control and the treated groups is assessed by analysis of variance with subsequent inter group comparison by Student's *t*-test with Bonferroni correction. $P < 0.05$ is required for significance.

MODIFICATIONS OF THE METHOD

Hossmann (1982) reviewed the experimental models of cerebral ischemia.

Yamamoto et al. (1992) studied the inhibition of NO biosynthesis on the volume of focal ischemic infarction produced by occlusion of the middle cerebral artery in spontaneously hypertensive rats.

Shigeno et al. (1985) described a recirculation model following middle cerebral artery occlusion in rats. The trunk of the middle cerebral artery was isolated between the rhinocortical branch and the lenticulostriate artery and encircled with a loose-fitting suture of nylon thread. The thread was exteriorized through a small polyethylene catheter, which was previously introduced into the craniectomy site through a burr hole in the zygoma. The artery was occluded by retraction of the thread, which was then fixed with biological glue. Recirculation was achieved by cutting and removing the thread.

Bederson et al. (1986a) occluded the middle cerebral artery at different sites in six groups of normal rats and characterized the anatomical sites that produce uniform cerebral infarction. A neurological system was developed that can be used to evaluate the effects of cerebral ischemia.

Bederson et al. (1986b) evaluated the use of 2,3,5-triphenyltetrazolium chloride as a histopathological stain for identification and quantification of infarcted brain tissue after middle cerebral artery occlusion in rats.

Yang et al. (1992) found a reduction of Na,K-ATPase activity in the ischemic hemisphere shortly after middle cerebral artery occlusion in rats.

Du et al. (1996) induced transient focal cerebral ischemia in rats by a 90 min period of ligation of the right middle cerebral artery and both common carotid arteries.

Germano et al. (1987) found a decrease of stroke size and deficits in rats with middle cerebral artery occlusion after treatment with kynurenic acid, a broad spectrum antagonist of excitatory amino acid receptors.

Gotti et al. (1988) found a reduction of the volume of infarcted tissue due to occlusion of the middle cerebral artery in **rats** and **cats** after administration of NMDA receptor antagonists.

Hossmann and Schuier (1980) studied experimental brain infarcts in **cats** after occlusion of the left middle cerebral artery.

Retro-orbital occlusion of the middle cerebral artery in cats was performed by Sundt and Waltz (1966) and by Gotti et al. (1988).

Welsh et al. (1987), Backhauß et al. (1992) described focal cerebral ischemia in **mice** after permanent occlusion of the middle cerebral artery.

Hara et al. (1997) found a reduction of ischemic and excitotoxic neuronal damage by inhibition of interleukin 1β-converting enzyme family proteases after occlusion of the middle cerebral artery in **mice** and **rats**. Nylon filaments were introduced from the carotid artery which were withdrawn after 2 h. One day later, the animals were tested for neurological deficits and the brains analyzed for infarct size and interleukin 1β levels.

Huang et al. (1994) produced **knock-out mice** deficient in the neuronal isoform of NO synthase by targeted disruption of the neuronal nitric oxide synthase gene. In these mice, Hara et al. (1996) found reduced brain edema and infarction volume after transient middle coronary artery occlusion.

Nishimura et al. (1998) described an experimental model of thromboembolic stroke without craniotomy in **cynomolgus monkeys** by delivering an autologous blood clot to the middle cerebral artery. A chronic catheter was implanted in the left carotid artery in male cynomolgus monkeys. A 5 cm long piece of an autologous blood clot was flushed into the internal carotid artery with physiological saline. A neurological score was assigned at 0.167, 0.5, 1, 2, 4, 6, and 24 h after embolization. In the acute phase after embolization, typical behavior consisted of circling gait and moderate deviation towards the side of embolization, long-lasting and strong extensor hypotonia of the contralateral lower and upper limbs, and mild to severe incoordination. Contralateral hemiplegia was observed over the following 24 h. At 24 h the animals were sacrificed immediately after the last neurological scoring, and the cerebral vasculature was inspected for the location of the clot. The brain was then cut into 2 mm thick coronal sections. Cerebral infarction size and location were ascertained by the triphenyl-2H-tetrazolium chloride staining method. The lesions involved mostly the caudate nucleus, internal capsule, putamen and thalamus.

Salom et al. (1999) subjected female **goats** to 20 min global cerebral ischemia under halothane/N_2O anes-

thesia. An episode of transient global ischemia was achieved by occlusion of the two external carotid arteries and simultaneous external compression of the jugular veins by a neck tourniquet. A reperfusion period started when the occlusions were released, and it was monitored for 2 h.

De Ley et al. (1988) studied experimental thromboembolic stroke induced by injection of a single autologous blood clot into the internal cerebral artery in dogs by **positron emission tomography**.

Functional magnetic resonance imaging has been developed as **pharmacological magnetic resonance imaging** for pharmacodynamic assays and to establish brain-penetrating parameters (Leslie and James 2000). Although regional cerebral metabolic rate of glucose is strongly increased during cerebral activity, the cerebral metabolic rate of O_2 is not increased in direct proportion. The result is that the relative uptake of O_2 from blood actually decreases. The veins and capillaries draining from the activated region are 'arterialized' and their deoxyhemoglobin concentration is reduced. Deoxygenated and oxygenated hemoglobin have different magnetization properties; thus the changes in the ratio of these two entities can be detected by blood-oxygen-level-dependent (BOLD) magnetic resonance imaging. At least for studies in animals, these methods may be preferred to positron emission tomography (Cherry and Phelps (1996).

Edema following middle cerebral artery occlusion in spontaneously hypertensive rats was measured by magnetic resonance imaging (Seega and Elger 1993; Elger et al. 1994a). Magnetic resonance imaging was also used to determine the size of intracerebral hem-orrhage in rats induced by stereotactic microinfusion of collagenase into the caudate putamen (Elger et al. 1994b).

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A.8.1.5 Photochemically induced focal cerebral ischemia in rats

PURPOSE AND RATIONALE

Focal cerebral ischemia in rats can be induced by irradiation with intensive green light via a fibre optic through the skull after injection of the dye Rose Bengal. The resulting cerebral infarct can be studied for various parameters, such as infarct volume, water content, local cerebral blood flow and glucose utilization.

PROCEDURE

Male Sprague-Dawley rats (300–350 g) are used. Anesthesia is induced with 3% halothane in oxygen and is maintained with 1% halothane in oxygen applied via a face mask. A small incision is made in the skin over the right femoral vein and a thin catheter is inserted. One ml of the dye Rose Bengal (in a concentration of 5 mg/ml of saline) is injected. A midline head incision is made and the right side of the skull is exposed. An intense green light, produced by a xenon lamp (75W, Zeiss, FRG) and then passed through a filter (wavelength 570 nm, Schott, Mainz, FRG) and a heat filter (Schott, Mainz, FRG), is directed on the skull at the level of the bregma for 15 min. The 3 mm diameter of the illuminated circle is determined by passing the light through a fibre optic (Schott, Mainz, FRG) while in close contact to the skull. The temperature of the skull underneath the fibre optic does not change during the time of illumination. At the end of the induction period, the temporary catheter is removed from the femoral vein which remains patent following the closure of the catheterization site with liquid suture (Histoacryl, Braun, Melsungen, FRG), and the incisions in the leg and head are likewise sutured after liberal application of local anesthetic. The anesthetic gas mixture is discontinued and the rats are allowed to recover consciousness in a warm environment until such times as the appropriate experiment is to be performed. Due to the non-invasive nature of this technique it is not possible to measure blood pressure or blood gases during the ischemic period. However, rectal temperature and plasma glucose concentrations are controlled.

Measurement of infarct volume

Osmotically controlled mini-pumps (Model 2ML1 Alzet®, USA) are placed into the peritoneum of two groups of six male Sprague Dawley rats (body weight 300–350 g). The mini-pumps are fitted via thin polyethylene catheters to the femoral vein of the rats. Each pump contains either 2 ml of physiological saline or 2 ml of a solution of the drug to be studied. The animals are then given an ischemic insult as described in the previous section. Seventy-two hours after the induction of ischemia, the rats are sacrificed by decapitation and the brains removed and frozen at –50 °C. Coronal sections (20 μ) are cut in a cryotome at –20 °C, fixed in Haidenhain's Susa and stained with Cresyl Violet. The ischemic area on 90 sections is measured and the volume of ischemic change is then calculated using a linear trapezoidal extrapolation of the areas measured.

Measurement of brain water content

Three groups each of six rats are used. One group receives no ischemic lesion (the illumination with green light is omitted from the experimental protocol) and two groups are lesioned as described above. One lesioned group receives the test drug orally at 15 min, 30 min, 1, 3 and 5 h after the induction of the ischemia or sham operation. The other group is treated with saline at the same time-points. Twenty-four hours later the rats are sacrificed by decapitation. The brains are rapidly removed and placed on a cutting block with 1 mm gradations. Two cuts are made 1 mm or less anterior and posterior to the lesion. The thick section so produced is then divided into left (non-lesioned) and right (lesioned) halves and placed in pre-weighed vials and the wet weights of the tissue samples are carefully measured. The tissue is then frozen in liquid nitrogen and then left under vacuum (less than 0.1 Torr) for 24 h. On removal the vials are sealed to prevent rehydration and reweighed to obtain the dry weight of tissue from which the water content (expressed as percentage of wet weight of tissue) is calculated.

EVALUATION

All data are presented as mean ±SD of the mean. For left (contralateral to the lesion) against right (ipsilateral to the lesion) comparisons, a *t*-test with paired com-

parison was used ($p < 0.05$). Statistical differences between groups were calculated using the unpaired *t*-test.

MODIFICATIONS OF THE METHOD

Boquillon et al. (1992) produced cerebral infarction in mice by intravenous injection of 10 mg/kg rose bengal, and by focal illumination of the intact skull surface for 3 min with a laser source, operating at 570 nm with power levels of 2, 5, 10, and 20 mW.

Matsuno et al. (1993) used a similar model to induce cerebral ischemia in rats based on middle cerebral artery occlusion by the photochemical reaction of rose bengal after irradiation with high intensity green light.

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A.8.1.6 Microdialysis and neuroprotection experiments after global ischemia in rats

PURPOSE AND RATIONALE

Transient global ischemia can be induced in rats by electrocauterization of the vertebral arteries followed by clamping of the carotid arteries.

PROCEDURE

Male Wistar rats weighing 280–340 g are used. The rats are anesthetized with methohexital sodium (60 mg/kg i.v.) and the vertebral arteries are electrocauterized (Pulsinelli and Brierley 1979). The rats are fasted overnight and re-anesthetized on the following day with halothane and intubated. The femoral artery and vein are cannulated to allow blood sampling, blood withdrawal and recording of mean arterial pressure (MAP). Samples of blood are taken at regular time intervals and blood gas/acid base status is analyzed (Instrumentation Laboratory, 1306). Rectal temperature is measured with a thermistor and controlled at 37 °C by means of a heating lamp. Four-vessel occlusion ischemia is induced for 20 min by bilateral carotid clamping followed by a period of reflow.

Microdialysis experiments

The head of the rat is fixed in a stereotactic frame. The skin is incised over the head, pulled apart and a 3×3 mm hole is drilled through the cranium. A microdialysis probe (Sandberg et al. 1986) is implanted into CA1 region of the hippocampus (2.2 mm lateral and 3.8 mm dorsal to bregma, and the window of the dialysis membrane 1.4–2.9 mm below the cortical surface). The electroencephalogram (EEG) is measured continuously with a tungsten electrode attached to the dialysis probe. The probe is perfused at a rate of 2.5 µl/min with a modified Ringer solution. Dialysates are sampled every 10 min and analyzed for purines (Hagberg et al. 1987) and amino acids (Lindroth et al. 1985). One group receives the test drug i.p. 15 min prior to ischemia whereas the control group obtains saline. The animals are followed during 20 min of ischemia and 2 h of reflow.

HPLC analyses are carried out using a reverse-phase C_{18} column (Waters 10 µm µBondapak) with isocratic elution, a flow rate of 1.0 ml/min and at ambient temperature. For adenosine, inosine and hypoxanthine the mobile phase is 10 mM $NH_4H_2PO_4$, pH 6.0, 13% methanol.

Neuroprotection experiments

During ischemic insult and for 20 min of reflow, the temperature of the temporalis muscle is controlled at 37 °C. Immediately following ischemia the rats are divided into two groups. One group is treated with the test drug and the other with saline. A bolus injection is administered i.p. 15 min after ischemia and a mini-osmotic pump is implanted into the abdominal cavity which delivers the test drug for 7 days. Control animals receive a bolus of saline and mini-osmotic pumps filled with saline. Seven days later the rats are anesthetized with pentobarbital and perfusion-fixed with formol saline. The histological evaluation is done "blind". The hippocampal damage is semiquantified according to the following scoring system:

0 = no damage
1 = scanty damage,
2 = moderate damage
3 = severe damage
4 = complete loss of pyramidal cells in the hippocampus.

EVALUATION

The purine and amino acid data are expressed as means ±SEM and differences are evaluated with the non-parametric Mann-Whitney test. The neuroprotective efficacy of the test drug is evaluated with two-tailed Student's *t*-test.

MODIFICATIONS OF THE TEST

In addition to the assessment of neuronal damage, Block et al. (1996) tested spatial learning of treated rats one week after 4-vessel occlusion in a Morris water maze.

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A.8.1.7 Hypoxia/hypoglycemia in hippocampal slices

PURPOSE AND RATIONALE

The *in vitro* release of adenosine, inosine, and hypoxanthine from rat hippocampal slices can be determined with and without drug.

PROCEDURE

Male Sprague-Dawley rats weighing 150–275 g are decapitated, the hippocampi isolated and cut into 400 µm thick slices which are placed into KRB (4 °C) containing (mM) NaCl (118), KCl (4.85), $MgSO_4$ (1.15), KH_2PO_4 (1.15), $NaHCO_3$ (25), glucose (5.5), $CaCl_2$ (1.3) equilibrated with 95% O_2/5% CO_2. The incubation medium is brought up to room temperature over a period of 30 min. The KRB is then replaced with fresh medium and the slices are incubated for a further 30 min at room temperature, followed by 30 min at 37 °C in fresh KRB. Following the initial incubations, slices are labelled for 45 min with ^{3}H-adenine (5 µCi/ml) at 37 °C. Two labelled slices are transferred into plastic cylinders which have nylon net bases and these, together with the slices, are placed into glass superfusion chambers. Slices are superfused at a flow rate of 0.5 ml/min with KRB at 37 °C. After a 60 min wash, collection of 5 min fractions begins, which continues throughout the remainder of the experiment. A 1.25 ml aliquot of the fractions is taken for determination of radioactivity using scintillation spectrometry (scintillation fluid: Picofluor 15). The remaining 1.25 ml is taken for HPLC analysis of purines and amino acids.

Hypoxia/hypoglycemia is induced by superfusion with KRB containing no glucose and 95% N_2 and 5% CO_2 for 35 min followed by recovery. All other procedures are as described above. The test drug is added to the perfusion fluid at an appropriate concentration. The 1.25 ml aliquots taken for HPLC analysis are pooled with two other aliquots (total 3.75 ml), lyophilized and concentrated 10 fold before analysis. Samples are analyzed for adenosine, inosine and hypoxanthine with HPLC. The radioactivity associated with each of these fractions is also determined by collecting the eluent from the column at the appropriate times. HPLC- analyses are carried out using a reverse-phase C_{18} column (Waters 10 µm µBondapak) with isocratic elution, a flow rate of 1.0 ml/min and at ambient temperature. For adenosine, inosine and hypoxanthine the mobile phase is 10 mM $NH_4H_2PO_4$, pH 6.0, 13% methanol.

EVALUATION

Values for adenosine, inosine and hypoxanthine can be expressed in two ways: (1) release rate per slice (pmol/min × slice); (2) percentage of the total amount of released radioactivity (% total $_3$H-label released). The purine data are expressed as means ±SEM and differences are evaluated with the non-parametric Mann-Whitney test.

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A.8.1.8 Measurement of local cerebral blood flow and glucose utilization in rats

PURPOSE AND RATIONALE

Cerebral glucose utilization can be determined using [^{14}C]-2-deoxyglucose according to Sokoloff et al. (1977). Local cerebral blood flow using [^{14}C]-iodoantipyrine is measured as described by Sakurada et al. (1978).

PROCEDURE

Animal preparation

The experiments are performed on male Sprague-Dawley rats (260–300 g). Catheters are placed, under light halothane (1%) anesthesia, into the femoral vein and artery of each hind limb (for the measurement of mean arterial pressure, the sampling of arterial blood and the intravenous administration of drugs and radioisotopic tracer). The wound sites are then infused with local anesthetic, sutured, and protected by pads. The lower abdomen is covered by an elastic stocking, followed by a loose-fitting plaster coat. Anesthesia is discontinued and the rats are allowed at least two hours to recover before any further manipulations are performed.

Experimental protocol

The rats are given an intravenous infusion (50 µl/min) of either saline or the test drug dissolved in saline. This infusion is maintained throughout the measurement of local cerebral blood flow or local cerebral glucose utilization.

Local cerebral glucose utilization

A full description of the method for measuring local cerebral glucose utilization using [^{14}C]-2-deoxyglucose has been published (Sokoloff et al. 1977). Five minutes after the administration of the test drug, the experiment is started with the intravenous administration of [^{14}C]-2-deoxyglucose (125 µCi/kg). Fourteen timed arterial blood samples are taken during the following forty-five minutes. These samples are centrifuged and the plasma is measured for glucose concentration (using an automated glucose analyzer) and [^{14}C] levels (by liquid scintillation counting). At the end of this period the rats are sacrificed by decapitation, the brain rapidly removed and frozen at –45 °C. Twenty micron thick coronal sections are cut in a cryostat (–22 °C) and autoradiograms are prepared by placing these sections in an array against Kodak SB-5 X-ray film along with pre-calibrated plastic standards range (55–851 nCi/g) for seven days in light-tight cassettes.

Local cerebral blood flow

The autoradiographic measurement of local cerebral blood flow using [^{14}C]-iodoantipyrine is carried out as described by Sakurada et al. (1978). [^{14}C]-iodoantipyrine (125 µCi/kg) is administered fifteen minutes after the infusion of the test drug has commenced. In a period of sixty seconds, eighteen timed arterial samples are collected in pre-weighed filter-paper discs from a free flowing arterial catheter. The discs are reweighed and the [^{14}C] concentration of each is measured by liquid scintillation counting. At the end of one minute, the rat is decapitated and autoradiograms are prepared in the same manner as for the measurement of local cerebral glucose utilization.

Densitometric analysis of autoradiograms

Tissue [^{14}C] concentrations were determined using a densitometer system (Zeiss, FRG) by reference to the images of the precalibrated standards. For each structure of interest, bilateral determination of optical densities are made on six different autoradiographic images in which the structure is best defined.

The mean optical density is used to calculate [^{14}C] concentrations. From this value, and the history of [^{14}C] in the blood, values of local cerebral blood flow and glucose utilization are obtained using the respective operational equations of these methods (Sakurada et al. 1978; Sokoloff et al. 1977).

EVALUATION

Groups of data are statistically compared by *t*-test with unpaired comparison using the BONFERRONI correction factor for multiple group analyses. Linear regression data for comparing cerebral blood flow (CBF) and glucose utilization (GU) undergo log transformation as recommended by McCulloch et al. (1982).

MODIFICATIONS OF THE METHOD

Ito et al. (1990) measured glucose utilization in the mouse brain by the simultaneous use of [^{14}C]2-deoxyglucose and [^{3}H]3-O-methylglucose.

High-resolution animal positron emission tomography (PET) was recommended by Magata et al. (1995) for noninvasive measurement of cerebral blood flow with ^{15}O-water and glucose metabolic rate with ^{18}F-2-fluoro-2-deoxy-glucose.

The effect of ginseng pretreatment on cerebral glucose metabolism in ischemic rats using animal positron emission tomography (PET) with [^{18}F]-2 fluoro-2-deoxy-D-glucose ([^{18}F]-FDG) was described by Choi et al. (1997).

Hawkins et al. (1993) developed a method for evaluating tumor glycolytic rates *in vivo* with nude mice injected with 2-[^{18}F]fluoro-2-deoxy-D-glucose and a dedicated animal positron emission tomography scanner.

Positron emission tomography has been used with specific ligands for CNS imaging (de la Sayette 1991; Jones et al. 1991; Kung 1993).

Rogers et al. (1994) synthesized ^{18}F-labelled vesamicol derivates to be evaluated in small animal positron emission tomography.

Hume et al. (1997) measured *in vivo* saturation kinetics of two dopamine transporter probes using a small animal positron emission tomography scanner.

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A.8.1.9 Cerebrovascular resistance in anesthetized baboons

PURPOSE AND RATIONALE

Cerebral blood flow and cerebrovascular resistance can be measured by injection of inert radioactive gas (^{133}Xenon) and evaluation of the ^{133}Xe clearance curve in anesthetized baboons.

PROCEDURE

Animal preparation

The experiments are performed on baboons (*Papio anubis* or *cynocephalus*) weighing 9–13 kg. Initial sedation with phencyclidine (10 mg i.m.) is followed by an intravenous injection of sodium thiopental (75 mg/kg). The animals are intubated to a positive pressure ventilator delivering 70:30% nitrous oxide and oxygen in open circuit. A continuous intravenous infusion of phencyclidine (0.01 mg/kg/min) is given throughout the course of the experiment. Suxamethonium (50 mg i.m.) is administered every 30 min in order to assist control of ventilation with the respiratory pump.

During the experiments, the end tidal concentration of CO_2 is continuously measured and the ventilating pump adjusted to maintain an arterial CO_2 tension ($PaCO_2$) of between 37 and 40 mm Hg for the control measurements. Arterial blood samples are taken during every measurement of cerebral blood flow (CBF) to measure $PaCO_2$, PaO_2, and pH by direct reading electrodes (Corning). Body temperature is maintained

around 37 °C by means of an electrically heated blanket and infrared heating lamps.

Catheters are inserted into the aorta via femoral arteries for the continuous measurement of arterial blood pressure and for the withdrawal of arterial blood samples. Both femoral veins are cannulated, one for the continuous infusion of phencyclidine and the other for the infusion of the test drug. A catheter is inserted into the right lingual artery so that its tip lays just distal to the carotid bifurcation. This catheter is flushed at regular intervals with heparinized saline to prevent platelet aggregation at the tip. In the studies with required intravenous administration of test drug, the other branches of the external carotid artery are ligated. Where the requirement is for intracarotid administration of test drug, another catheter is retrogradely advanced into the external carotid artery so that its tip lays next to the tip of the lingual catheter. This catheter is then attached to a constant-rate infusion pump (Sage Instruments). Heparinized saline is infused at a rate of 0.2 ml/min to act as a control for drug infusion.

A burr hole is made over the midline fissure and a catheter inserted into the sagittal sinus for the withdrawal of cerebral venous blood samples. The hole is sealed with plaster of Paris. The scalp and temporalis muscle are removed with diathermy down to the level of the zygomatic arch.

Measurement of cerebral blood flow, cerebral oxygen utilization, and cerebrovascular resistance

A collimated scintillation crystal is placed over the temporal region of the exposed skull on the right side and angled in such a way that viewed only brain and overlying skull.

Approximately 260 μCi of ^{133}Xenon dissolved in 0.5 ml sterile heparinized saline (500 IU) is injected over 1 s into the catheter in the lingual branch of the carotid artery. The gamma-ray emission of the $_{133}$Xe are detected by the scintillation counter attached to a photomultiplier. The pulses are amplified and subjected to pulse height analysis (peak 81 KeV ±8 KeV) to reduce Compton scatter before fed into a rate meter and scaler. The output from the rate meter is displayed on a chart recorder. Cerebral blood flow is calculated from the height/area equation (Høedt-Rasmussen et al. 1966). The formula used is

$$F = (H_1 - H_{10}) \times 100 / \lambda A_{10},$$

where F is CBF in ml blood per 100 g brain tissue per min; λ = brain tissue/blood partition coefficient for ^{133}Xe (the figure of 1.1 is used [Veall and Mallett 1966]); H_i = maximum initial height of the ^{133}Xe clearance curve in counts per min as taken from the chart recorder; H_{10} = height of the clearance curve 10 min after the peak height in counts per s; A_{10} = total integrated counts over the 10 min of clearance as taken from the scaler and corrected for background activity over that period.

Cerebral oxygen consumption is measured from the product of the DBF and the difference in oxygen content between the arterial blood and cerebral venous blood sampled from the sagittal sinus. Blood oxygen is measured by a charcoal-fuel cell system (Lex-O_2-Con).

An estimate of cerebrovascular resistance is obtained by subtracting the mean sagittal sinus pressure from the mean arterial pressure and dividing this pressure difference by the CBF.

EEG recording

Electroencephalographic readings are recorded bilaterally throughout the experiment. A series of holes are drilled in the calvarium 10 mm apart in two rows. Each row is 14 mm lateral to the sagittal suture. The holes are threaded to receive nylon screws in which silver-silver chloride ball electrodes are fixed loosely. The electrodes are positioned epidurally and the free ends are soldered to a multi-channel socket which is mounted on the calvarium with plaster of Paris.

Experimental procedure

Following completion of the surgery, the animals are left undisturbed for 1 h. At least three control estimations of CBF and other parameters are made until steady conditions of flow, arterial blood pressure, and blood gas tensions are obtained.

Protocols

Intravenous administration. After stable control values have been established, the infusion is started. The CBF is measured at 5 min after the start and again at 25 min. The infusion is stopped 10 min after this flow period, giving a total infusion time of 35 min. Further flow measurements are made at 10, 30, and 50 min after stopping the infusion.

Intracarotid administration. After establishing control values, the intracarotid infusion of the test drug is begun. The CBF is measured at 10, 30, 50 and 70 min. The infusion is stopped 80 min after commencing, and post infusion measurements are made after 20, 40, and 60 min.

EVALUATION

Data are presented as mean values ±SEM Evaluation of statistical significance is performed by means of Student's *t*-test with Bonferroni correction.

MODIFICATIONS OF THE METHOD

Kozniewska et al. (1992), Wang et al. (1992) measured cerebral blood flow in **rats** by intracarotid injection of ^{133}Xe.

Delayed cerebral vasospasm was induced in anesthetized **dogs** by removal of 4 ml cerebrospinal fluid and injection of the same volume of fresh autologous arterial nonheparinized blood into the cisterna magna by Varsos et al. (1983) as a model of subarachnoid hemorrhage. The procedure was repeated on the third day and angiograms were taken of the vertebral-basilar vessels. The reduction of diameter of the basilar artery was taken as endpoint.

Imaizumi et al. (1996) produced experimental subarachnoid hemorrhage by intracisternal injection of arterial blood in **rabbits**. The degree of vasospasm and the effect of calcitonin gene-related peptide were evaluated angiographically by measuring the basilar artery diameter.

Inoue et al. (1996) produced experimental subarachnoid hemorrhage in **cynomolgus monkeys** by placing a clot around the internal carotid artery. A series of angiographic analyses were performed, before subarachnoid hemorrhage and on days 7 and 14 after treatment with calcitonin gene-related peptide to examine changes in the diameter of the ipsilateral internal carotid artery, middle cerebral artery, and anterior cerebral artery.

Hughes et al. (1994) adapted the ^{133}xenon clearance technique for simultaneous measurement of cutaneous blood flow in **rabbits** at a large number of skin sites within the same animal.

Solomon et al. (1985), Clozel and Watanabe (1993) induced cerebral vasoconstriction by injection of autologous blood in the cisterna magna of rats. Cerebral blood flow was measured with the radioactive microsphere technique.

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A.8.1.10 Effect on cerebral blood flow in cats (Fluvography)

PURPOSE AND RATIONALE

Cerebral blood flow in anaesthetized cats an be measured by fluvography. The heat transfer coefficient of the brain can be measured with a probe representing an indirect blood flow value.

In this method, the measuring device consists of a thermo probe, which is attached to the tissue in order to record continually the heat transport (Hensel, 1956). The device depends on having an electrically heated part and an unheated reference point. The difference in temperature between the heated part of the device and the unheated reference point is a function of local blood flow. An increase in flow tends to lower the local temperature by carrying away the heat gain and vice versa.

PROCEDURE

Cats of either sex weighing 2.5–4.0 kg are anaesthetized by intraperitoneal administration of pentobarbital sodium (35 mg/kg) and intubated with a tracheal tube. The left femoral vein and the right femoral artery are cannulated for i.v. drug administration and determination of arterial blood pressure, respectively. The arterial cannula is connected to a Statham transducer P 23 Db.

For intraduodenal drug administration the duodenum is cannulated following laparotomy.

Before actually starting the experiment, the arterial blood gas concentrations are determined.

Animals are only used for further testing if they show normal blood gas concentrations. During the course of the experiment, blood flow, blood pressure and blood gas concentrations are regularly monitored.

The head of the animal is fixed in a stereotactic device. The skull cap and the dura are opened, the probes are placed on the surface of the cortex in the region of the marginal frontal gyrus, and the exposed brain is covered with moist swabs. The Fluvograph (Hartmann + Braun, Frankfurt) is used with the appropriate thermo probes.

To test the correct position of the thermocouple and the response of the animal, inhalations of carbon dioxide/ air or injections of epinephrine (adrenaline) are used, leading to a distinctive increase in cerebral blood flow. Following stabilization of the parameters mentioned above, the standard compound is administered and the change in blood flow is recorded. Five min after obtaining the original values, the test compound is administered.

Standard compound:

- pentoxifylline
  1 and 3 mg/kg (i.v. administration)
  10 and 30 mg/kg (i.d. administration)

EVALUATION

The percent change in the heat transfer coefficient is used as an indirect measure for the change of cerebral blood flow. Statistics: Student's *t*-test is performed by unpaired comparison.

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A.8.1.11 Effect on cerebral blood flow and in ischemic skeletal muscle in rats (Laser-Doppler-Effect)

PURPOSE AND RATIONALE

The principle of the Laser Doppler effect is based on the fact that a laser light beam directed on tissue is scattered in static structures as well as in moving cells. Light beams scattered in moving red cells undergo a frequency shift according to the Doppler effect, while beams scattered in static tissue alone remain unshifted in frequency. The number of Doppler shifts per time is recorded as a measure for erythrocyte flow in a given volume. This means, that the direction of flow cannot be determined, but relative changes in micro circulatory blood flow can be recorded. The procedure can be used to detect test compounds that improve the blood supply of the brain or the flow of red blood cells in the ischemic skeletal muscle.

PROCEDURE

Male Sprague-Dawley rats weighing 300–500 g are anaesthetized with pentobarbital sodium (60 mg/kg i.p.). The trachea is exposed and intubated with a short tracheal tube to allow ventilation. The following vessels have to be cannulated: The femoral vein is cannulated for test drug administration. The femoral artery is cannulated for blood pressure recording and blood gas analysis.

Before actually starting the experiment, the arterial blood gas concentrations are determined. Animals are only used for further testing if they show normal blood gas concentrations (pa CO_2: 32–42 mm Hg; pa O_2: 70–110 mm Hg). The mean arterial blood pressure should not drop below 100 mm Hg. During the course of the experiment, blood flow, blood pressure and blood gas concentrations are regularly monitored.

For cerebral blood flow

The head of the animal is fixed in a stereotactic device. After trepanation of the skull (opening 3 mm in diameter), the Laser Doppler probe is placed 1 mm above the surface of the brain. Values are measured with the Laser Doppler apparatus (Periflux F2, Perimed KB, Stockholm).

Following stabilization of the parameters mentioned above, the standard compound is administered and the change in blood flow is recorded. Five min after obtaining the original values, the test compound is infused. Following two administrations of the test compound, the standard compound is administered again. Duration of the effect is measured as half life in seconds.

For peripheral blood flow

A small area of the femoral artery of the right hind limb is exposed and the Laser Doppler probe is placed 1 mm above the muscle surface. Before actually starting the experiment, the arterial blood gas concentrations are determined. Animals are only used for further proceeding if they show normal blood gas concentrations. During the course of the experiment, the blood pressure is recorded. The RBC flux is recorded

continuously and after stabilization of the output signal, the femoral artery is occluded leading to underperfusion of the muscles of the right pelvic limb. At this stage, the test compound is administered by intravenous infusion for 10 min (0.05 ml/min).

Standard compound for cerebral blood flow is propentofylline (1 mg/kg, i.v.). The percent increase in blood flow produced by propentofylline ranges between 40% and 60%.

EVALUATION

The percent increase in blood flow after test drug administration is determined (compared to the value before drug administration).

Statistics: Student's *t*-test by unpaired comparison, test substance versus standard.

MODIFICATIONS OF THE METHOD

Iadecola (1992), Prado et al. (1992), Raszkiewicz et al. (1992) measured the influence of nitric oxide on cortical cerebral blood flow in anesthetized rats by Laser-Doppler flowmetry.

Benessiano et al. (1985) measured aortic blood flow with range-gated Doppler flowmeter in anesthetized rats.

Partridge (1991) measured nerve blood flow in the sciatic nerve of anesthetized rats with a Laser Doppler Flowmeter after application of local anesthetics and of epinephrine.

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A.8.1.12 Traumatic brain injury

PURPOSE AND RATIONALE

A major goal in research into mechanisms of brain damage and dysfunction in patients with severe head injury and in discovery of potential therapies is the development of a suitable animal model. While a variety of experimental techniques have been developed (Denny-Brown and Russell 1941; Gurdjian et al. 1954; Ommaya and Gennarelli 1974; Sullivan et al. 1976; Nilsson et al. 1977; Hayes et al. 1984; Marmarou et al. 1994; Mésenge et al. 1996), the most widely employed technique is fluid percussion, which produces brain injury by rapid injection of fluid into the closed cranial cavity (Sullivan et al. 1976; McIntosh et al. 1989, 1990; Faden et al. 1989; Dixon et al. 1991; Sun and Faden 1995; Petty et al. 1996). Most authors used rats, however, other species, such ad cats (Sullivan et al. 1976; Hayes et al. 1983, 1984), rabbits (Lindgren and Rinder 1969) and mice (Hall et al. 1988; Mésenge et al. 1996) were employed.

PROCEDURE

Surgical preparation

Male Sprague Dawley rats weighing from 400 to 500 g are anesthetized with ketamine (80 mg/kg, i.m.) and sodium pentobarbital (20 mg/kg, i.p.). During surgical preparation and throughout the experiment, all wounds are infused with a topical anesthetic (lidocaine hydrochloride 2.0%). Catheters are inserted into the femoral vein for drug administration and into the femoral artery for blood pressure/blood gas monitoring. A 2.0 mm hollow female Leur-Loc fitting (to induce trauma) is rigidly fixed with dental cement to the animal's skull in a craniectomy centered over the left parietal cortex 5 mm from lambda, 5 mm from bregma, 4 mm from sagittal suture. The dura is left intact at this opening. Immediately following surgical preparation, a constant i.v. infusion of sodium barbital (15 mg/kg/h) is begun and maintained for the duration of the studies.

Drug administration

Drugs or equal volumes of saline are administered through the femoral vein over 10 min by constant infusion beginning 15 min before trauma.

Fluid-percussion injury

The fluid-percussion device consists of a Plexiglas cylindrical reservoir, 60 cm long and 4.5 cm in diameter, bounded at one end by a Plexiglas, cork-covered

piston mounted on O-rings. The opposite of the reservoir is fitted with a 2-cm-long metal housing on which a transducer is mounted and connected to a 5-mm tube (2 mm inner diameter) that terminates with a male Leur-Loc fitting. At the time of injury the tube is connected with the female Leur-Loc fitting that has been chronically implanted over the exposed dura of the rat. After the entire system is filled with 37°C isotonic saline, injury is induced by a metal pendulum which strikes the piston of the device from a predetermined height. The device produces a pulse of increased intracranial pressure of fairly constant duration (21–23 ms) by injecting various volumes of saline into the closed cranial cavity. Brief displacement and deformation of neural tissue results from the rapid epidural injection of saline. The magnitude of injury is regulated by varying the height of the pendulum, which results in corresponding variations of the intracranial pressure expressed in atmospheres. The pressure pulses are measured extracranially by a transducer and recorded on a storage oscilloscope.

EVALUATION

Behavioral outcome

Posttraumatic deficits are evaluated at 24 h, 1 week and 2 weeks following trauma. Outcomes include forelimb flexion (right and left), lateral pulsion (right and left) and angle score (left, right and vertical position). Scores range from 0 (maximal deficits) to 5 (normal) for each task, By combining scores of all tests, a composite neuroscore is determined, ranging from 0 to 35 (Faden 1993).

Histopathology

At 2 weeks, following final neurological scoring, the rats are sacrificed by decapitation. The brain is removed, quickly frozen in isopentane and stored in a –80°C freezer until sectioning. Coronal brain sections are selected to span the longitudinal axis of the dorsal hippocampus between –3.2 and –3.8 Bregma. Sections (16 mm thick) are cut at –18°C in a microtome-cryostat and thaw-mounted onto chrome-gelatin rubbed microscope glass slides and kept at –80°C for histological study.

Sections are stained with Crystal violet. CA1 and CA3 pyramidal cells with a distinct nucleus and nucleolus are counted as viable neurons, in one reticle within CA3 and in three reticles (R1, R2 and R3) within the subfield of the hippocampus, in both right and left hemispheres. The number of viable neurons is counted twice at 400× microscope field.

An immunochemical method is used to detect glial fibrillary acidic protein (GFAP)-positive astrocytes in the hippocampus (Faddis and Vijayan 1988). Counting of the number of cells is done under 400× light microscopy in the dorsal CA1 subfield between medial and lateral regions.

Statistical analysis

Neuroscores from forelimb flexion tests, lateral pulsion tests and angle board tests are statistically analyzed by non-parametric Mann-Whitney U-tests. Histological data are analyzed by one way ANOVA test, followed by Scheffe's test.

MODIFICATIONS OF THE METHOD

Shohami et al. (1995) tested the effect of a non-psychotropic cannabinoid which acts as a non-competitive NMDA antagonist on motor and memory functions after closed head injury in the **rat**.

Fox et al. (1998) developed a **mouse** model of traumatic brain injury using a device that produces controlled cortical impact, permitting independent manipulation of tissue deformation and impact velocity and resulting in sustained sensory/motor and cognitive defects.

Tang et al. (1997) reported impairment in learning and memory in an experimental model of concussive brain injury in **mice**.

Bemana and Nago (1998) induced acute intercranial hypertension in **cats** by continuous inflation of an extradural balloon with physiological saline at a constant rate of 0.5 ml/h for 3 h. At this point, inflation was discontinued and the balloon remained expanded for an additional hour after which it was deflated.

A model of **traumatic injury to the spinal cord** was used by Springer et al. (1997). Female Long Evans rats weighing 200–250 g were anesthetized with pentobarbital and a dorsal laminectomy was performed to expose the spinal cord at thoracic level T 10. The vertebral column was stabilized by clamping the column at vertebra 8 and 11. The New York University (NYU) impactor device was used which produces accurate and reproducible damage to the rat spinal cord (Gruner 1992; Basso et al. 1996). This device is a weight drop apparatus that uses optical potentiometers to record the movement of a 10-g impact rod and the vertebral column following impact and is connected to a PC that monitors rod and vertebral movements during impact.

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A.8.2
Peripheral blood supply

A.8.2.1
Perfused hindquarter preparation with sympathetic nerve stimulation in rats

PURPOSE AND RATIONALE

Perfusion of the hindquarter in rats with a constant flow rate allows the evaluation of the effect of drugs on the peripheral vascular bed. Since constant blood flow is maintained, changes in the vascular resistance of the perfused bed are directly proportional to changes in the perfusion pressure.

PROCEDURE

Male Wistar rats weighing between 250 and 300 g are pre-treated with heparin (1 000 units/kg i.v.) and anesthetized with pentobarbital sodium (50 mg/kg i.p.) The animals are intubated with a tracheal tube and positive pressure ventilation is maintained with a Harvard Rodent Respirator at 4–6 ml/stroke and 50 strokes/min. The right jugular vein is cannulated with polyethylene tubing for administration of drugs.

The lumbar sympathetic chain is isolated dorsal to the inferior mesenteric branches via an abdominal midline incision. The aorta is freed from the vena cava and two silk ligatures are placed around the aorta. The aorta is ligated and cannulated proximal as well as distally with polyethylene tubings. A short piece of rubber tubing is inserted at the distal end to allow intraarterial injections of drugs. Two "T" junctions allow the measurement of arterial pressure and perfusion pressure by Statham P 23 Db pressure transducers being recorded through a Hellige physiological recorder. From the proximal part of the aorta, blood is forced to its distal part by a peristaltic pump (Desaga) through a glass coil kept at 40 °C. Flow rate is adjusted to produce a stable perfusion pressure as close to the systemic pressure as possible. After initial adjustment, flow rate is not altered for the remainder of the experiment.

Following perfusion pressure stabilization, the sympathetic chain is isolated and a small (1 mm wide, 2 mm long) curved bipolar electrode is placed around the nerve for electrical stimulation. Square-wave pulses from a Grass stimulator are used to activate the nerve with a constant current of 2.5 milliamps, supramaximal voltage and varying frequencies of 5 ms duration.

A dose-response curve is established for norepinephrine by giving doses of 0.01 µg, 0.03 µg, 0.1 µg, 0.3 µg, 1.0 µg, and 3.0 µg intra-arterially and measuring perfusion pressure changes. Similarly, a frequency-response curve to nerve stimulation is established by stimulation at 3 Hz, 6 Hz, and 10 Hz for 30 s. Two predrug readings are taken to insure consistent responses.

A minimum of four animals is used for each test compound.

EVALUATION

The first predrug dose-response curves are compared with the second predrug, 5 min and 60 min postdrug dose-response curves. From regression equations for norepinephrine and nerve stimulation, mean responses and potency values with 95% confidence limits are calculated.

MODIFICATIONS OF THE METHOD

Folkow et al. (1970) perfused the hindquarters of spontaneously hypertensive rats and control rats at a constant rate of flow with an oxygenated plasma substitute in order to study the increased flow resistance and vascular reactivity. The hindquarters were isolated from the upper part of the body by standardized mass ligatures at identical levels until the aorta and the inferior caval vein provided the only intact circulatory connections between the two parts of each animal.

Thimm et al. (1984) described reflex increases in heart-rate induced by perfusing the hind leg of the rat with solutions containing lactic acid.

Thimm and Baum (1987) obtained spike recordings from chemosensitive nerve fibres of group III and IV of the rat nervus peroneus. Applications were performed either by perfusion of the circulatory isolated hindleg or by superfusion of the isolated musculus extensor digitorum longus.

Kitzen et al. (1978) used the perfused hind limb of the **dog** with sympathetic nerve stimulation for cardiovascular analysis.

Reitan et al. (1991) developed a near anesthetic-free isolated hindlimb model in the **dog** and studied the effects of halothane and atropine sulfate on vascular resistance.

Wiegershausen and Deptalla (1969) used the isolated perfused hindlimb of the **cat** to study the influence of local anesthetics on the vasoconstrictor actions induced by bradykinin, epinephrine and histamine.

Santiago et al. (1994) analyzed the responses to bradykinin in the hindquarters vascular bed of the cat.

Champion et al. (1996, 1997) analyzed the responses of human synthetic adrenomedullin, an analog of adrenomedullin and calcitonin gene-related peptides in the hindlimb vascular bed of the cat.

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A.8.2.2 Effect on peripheral blood flow in rats

PURPOSE AND RATIONALE

Various methods exist to measure peripheral blood flow in rats, such as the microsphere technique, electromagnetic flowmetry and Doppler ultrasonic flowmetry.

Radioactive microspheres are used to calculate the distribution of blood from the heart to various organs and tissues before and after the administration of test compounds. In this method, microspheres are injected into the left cardiac ventricle. It is postulated that the first contraction of the cardiac muscle will expel these spheres into the circulation. Consequently the microspheres can be trapped in different organs according to the organ's perfusion rate. It is not a primary screening method but it is a useful test for distinguishing compounds with blood flow altering activities.

The microspheres used are 14 μ in diameter. They are marked with isotopes. In this test, four different radioactive elements are used (Cr^{51}, Sr^{85}, Sc^{46}, Ce^{141}), allowing the determination of blood flow before dosing and after the administration of 3 different compounds or 3 different doses (of the same compound).

PROCEDURE

Male Wistar rats weighing 500–550 g are anaesthetized with pentobarbital. The trachea is exposed and intubated with a short endotracheal tube to allow ventilation. Prior to testing the jugular vein is cannulated for administration of test drugs. The carotid artery is cannulated and later on the catheter is passed retrograde into the ventricle. Ventricular pressure is recorded to assure the correct emplacement of the catheter tip in the ventricle. This catheter is connected to another catheter allowing the injection of microspheres into the left ventricle later on. The right arteria brachialis is cannulated and connected to a Hellige blood pressure recorder. During the course of the test, blood pressure will be measured continuously. The left femoral artery is cannulated and connected to an infusion pump. During the experiment blood will be withdrawn from this artery.

After these preparations the rat is allowed 15–30 min to recover. Before actually starting the experiment, the arterial blood gas concentration of each animal is measured. Animals are only used for the experiment if they show normal blood gas concentrations. During the following procedure blood pressure, ventricular pressure and the heart rate are continuously recorded. To determine baseline blood flow animals receive 0.2 ml vehicle/min over a 3 min period. In the 4th minute rats receive the first injection of microspheres (Cr^{51}). Simultaneously 0.5 ml/min blood is withdrawn from the femoral artery catheter for one minute, the pump thus being used as a reference organ. The animals are allowed 20 min to recover before the administration of drugs. The test compound is infused into the jugular vein at a rate of 0.2 ml/min for 3 min followed by injection of the second microsphere (Sr^{85}). The same procedure is repeated using the other two microspheres (Sc^{46} and Ce^{141}) following administration of the second and third test compound.

At the end of the experiment blood gas concentrations are measured. The animals are killed and their organs are removed. Usually blood flow is determined in the following organs:

- brain (right and left hemisphere; right hemisphere showing slight ischemia due to cannulation of carotis)
- cerebellum
- lungs
- heart
- kidney (right and left)
- skeletal muscle (right hind extremity)
- duodenum
- stomach
- spleen
- diaphragm
- adrenal gland (right and left)

To determine effects of test compounds on the blood flow in the underperfused skeletal muscle the same experiment can be performed with the right femoral artery being clamped. In this way effects of the test drug on the ischemic and normal skeletal muscle (left thoracic limb) can be compared in the same animal.

EVALUATION

The rate of blood flow/tissue at a certain time is determined by measuring radioactivity in the different tissues and comparing the results to that of the blood sample.

MODIFICATIONS OF THE METHOD

Blood flow in various peripheral organs, e.g., renal blood, flow can be measured with electromagnetic flowmeters (e.g., Transflow 601, Skalar Medical, Holland) or with Doppler ultrasonic flowmetry (Shaffer and Medvedev 1991).

Lappe et al. (1986) studied regional vascular resistance in conscious spontaneously hypertensive rats

which were chronically instrumented with pulse Doppler flow probes to allow measurement of renal, mesenteric and hind quarters blood flow.

Hartman et al. (1994) validated a transit-time ultrasonic volume flow meter by simultaneous measurements with an electromagnetic flow metering method.

Lepore et al. (1999) used electron paramagnetic resonance to investigate the time course of nitric oxide generation and its susceptibility of nitric oxide synthase in ischemia-reperfusion injury to rat skeletal muscle *in vivo*. Total hind limb ischemia was applied for 2 h using a rubber band tourniquet method. At the end of ischemia the tourniquet was removed and the limb allowed to reperfuse for various time intervals.

Beattie et al. (1995) measured carotid arterial vascular resistance in anesthetized **rabbits**. Carotid blood flow was measured by a Doppler flow probe placed around the right common carotid artery. Dose-response curves of reduction of carotid arterial vascular resistance were constructed after injection of various doses of substance P-methyl ester via the right lingual artery. Intravenous injection of various doses of a selective tachykinin NK_1 receptor antagonist inhibited this effect dose-dependently.

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A.8.2.3
Effect on peripheral blood flow in anesthetized dogs

PURPOSE AND RATIONALE

Effects on blood pressure have to be analyzed whether they are mediated by central, cardial, or peripheral action. By injecting small doses of the test compound directly into a vascular bed, thus avoiding changes of central hemodynamics, peripheral vasodilating activity of a compound can be tested. This test is used to evaluate direct vasodilating or constricting activities of drugs *in vivo*-measurements of blood flow.

PROCEDURE

Male or female Beagle or Labrador-Harrier dogs weighing 15–25 kg are used. The dogs are premedicated intravenously with heparin (bolus of 500 IU/kg and successive injections of 50 IU/kg every 30 min) and anesthetized by intravenous injection of thiobarbital sodium (0.5 mg/kg i.v.), chloralose (20 mg/kg i.v.) and urethane (250 mg/kg i.v.). Respiration is maintained with room air through a tracheal tube using a positive pressure respirator. Blood gas analysis is performed at regular time intervals. Oxygen is supplied via the respirator as needed.

Preparation for hemodynamic measurements

To measure peripheral blood flow and to administer the test substance, a bypass is inserted into a femoral artery incorporating an electromagnetic flow probe and a port for injections. The other femoral artery is also equipped with a bypass used for the administration of a reference compound or a second test drug.

For recording of peripheral blood pressure and heart rate, one of the bypasses is connected to a pressure transducer (Statham P 23 BD).

All parameters are recorded continuously during the whole experiment.

Experimental course

When stable hemodynamic conditions are achieved for at least 20 min, the vehicle is administered (control), and 10 min later the test compound. Immediately after each administration, the port is flushed with physiological saline. Successive doses are administered after recovery to baseline values.

Readings are taken at times 0, 0.5, 1, 2, 5 and 10 min, and, if necessary, at additional 10 min-intervals following drug administration.

Standard compound:

- carbocromene 1 mg/kg

Characteristics:
- blood pressure
  - systolic, BPs
  - diastolic, BPd
- heart rate, HR
- peripheral blood flow, PF

EVALUATION

Changes in blood pressure, heart rate and peripheral blood flow at different times after drug administration are compared to vehicle control values obtained in the 10 min pre-drug period.

With $n > 2$, results are presented as mean ±SEM. Statistical significance is assessed by means of the paired *t*-test. Scores are compared to the efficacy of standard compounds for intensity and for duration of the effect.

MODIFICATIONS OF THE METHOD

Regional blood flow can be determined by the use of microspheres (Rudolph and Heyman 1967). The method is based on the principle that biologically inert microspheres will be trapped due their diameter in the microvasculature (Hales and Cliff 1977). The use of radioactive microspheres has some disadvantages (Buckberg et al. 1971). The use of fluorescent labeled microspheres for measurement of regional organ perfusion has been recommended (Glenny et al. 1993; Prinzen and Glenny 1994; van Oosterhout et al. 1995). Raab et al. (1999), Thein et al. (2000) described the automation of the use of fluorescent microspheres using a special sample processing unit. A Zymate-Robotic System (Zymark, Idstein, Germany) was modified to handle a special filtration device.

Ebara et al. (1994) measured renal blood flow in dogs after intrarenal arterial infusion of adrenomedullin.

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A.8.2.4 Effect on peripheral blood supply measured by local oxygen pressure

PURPOSE AND RATIONALE

Local oxygen pressure is directly related to oxygen supply to peripheral organs, e.g., muscle (Luebbers 1969; Kessler 1969). The local oxygen pressure (PO_2) is recorded directly on the muscle surface. In the following procedure the effect of test compounds on the local oxygen pressure (PO_2) of the normal and the ischemic skeletal muscle is determined.

PROCEDURE

Male Beagle dogs weighing 15–20 kg are used. The dog is anesthetized by intraperitoneal administration of pentobarbital sodium (Nembutal®). Prior to testing, the following vessels have to be cannulated: The V. femoralis of the left pelvic limb is cannulated for administration of test compounds. The A. femoralis of the left pelvic limb is cannulated for blood pressure recording. The V. femoralis of the right pelvic limb is cannulated. During the course of this test blood will be withdrawn from this vein to monitor lactate concentrations.

Small areas of muscles of the right pelvic limb and the right thoracic limb are exposed. Muscle relaxation is induced by intravenous injection of 0.1 mg/kg alcuronium chloride (Alloferin®) and maintained by i.p. administration of 0.05 mg/kg Alloferin at 30 min intervals. The trachea is exposed and intubated to assist the dog's respiration.

A PO_2 electrode is placed on the exposed muscle area of the right hind limb. After stabilization of PO_2 curves, the femoral artery of the right hind limb is occluded by putting a clip around the vessel. Muscle PO_2 drops rapidly. Following stabilization, test compounds are given by intravenous infusion for 10 min or by intraduodenal administration at this stage. The PO_2 of the non ischemic muscle is recorded simultaneously

via a second electrode on the right thoracic extremity. The clip is removed after maximally one hour. This procedure can be repeated up to four times in one animal. Blood gas concentrations and pH are determined at the beginning and end of each experiment.

Standard compound:
- pentoxifylline

EVALUATION
The following parameters are determined:

- Maximal increase in PO_2 (mm Hg) after administration of test drug
- duration of effect by determining the half life

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A.8.2.5 Effect on mesenteric blood flow in rats

PURPOSE AND RATIONALE
Blood flow in the mesenteric vascular bed *in situ* (Bhattacharya et al. 1977; Eikenburg 1984; Randall et al. 1989; Jackson and Inagami 1990) can be studied in rats in a way similar to that of the perfused hindquarter.

PROCEDURE
Wistar rats of either sex weighing 250–300 g are anesthetized with a combination of urethane (500 mg/kg i.v.) and sodium pentobarbitone (30 mg/kg). The abdomen is opened by a midline incision and a segment of the superior mesenteric artery is exposed by careful dissection of the surrounding tissue. Care is taken to avoid damage to the accompanying nerve terminals. One cannula is inserted into the carotid artery and the other into the superior mesenteric artery. Blood from the carotid artery is forced by a peristaltic pump (Desaga) into the superior mesenteric artery, using a glass coil kept at 40 °C. Blood required to fill the tubing initially is obtained from donor rats. Heparin is administered intravenously to the animals prior to cannulation of the mesenteric artery. The blood pressure and the perfusion pressure are measured by Statham P 23 Db pressure transducers and recorded through a 2-channel Hellige recorder. The pump speed is initially adjusted so that the perfusion pressure equals the systemic blood pressure. Intraarterial injections into the mesenteric vascular bed are made by puncturing the tubing going towards the periphery. Intravenous injections are made through a cannula inserted into the external jugular vein.

EVALUATION
Changes in the vascular resistance are measured by comparing perfusion pressure before and after drug administration. If constant blood flow is maintained, changes in the vascular resistance of the perfused bed are directly proportional to changes in the perfusion pressure.

MODIFICATIONS OF THE METHOD
Reactivity in the mesenteric vascular bed can be tested in an isolated preparation (McGregor 1965; Kawasaki and Takasaki 1984; Laher and Triggle 1984; McAdams 1984; Foy and Nuhu 1985; Longhurst and Head 1985; Soma et al. 1985; Hsueh et al. 1986; Longhurst et al. 1986; Manzini and Perretti 1988; Nassar et al. 1988; Randall and Hiley 1988). The abdomen of anesthetized rats is opened and the superior mesenteric artery is separated from surrounding tissue in the region of the aorta. A cannula is inserted into the superior mesenteric artery at its origin from the abdominal aorta. The cannula is filled with heparinized Krebs solution. The ileocoeliac branch of the artery is tied off and the intestine separated from the mesentery by cutting close to the intestinal border of the mesentery. The cannulated artery and its vascular bed are dissected out and mounted in an organ bath. The preparation is perfused with oxygenated Krebs-bicarbonate buffer (pH 7.4) at 37 °C. Perfusion pressure is recorded via a side arm of the arterial cannula using a Statham pressure transducer. The flow rate is adjusted to give a baseline perfusion pressure of 20–30 mm Hg. The test substances are infused into another side arm of the arterial cannula for 15 s using an infusion pump. After three stimuli with norepinephrine (1 µg) or potassium chloride (1 mg), the test drugs are infused followed by further stimulation. The inhibition of increase of perfusion pressure after test drugs is expressed as percentage of control.

Nuki et al. (1994) compared the vasodilating activity of chicken calcitonin gene-related peptide with human α-CGRP and rat CGRP in the precontracted mesenteric vascular bed of rats.

The **rabbit** isolated arterially perfused intestinal segment preparation was used by Brown et al. (1983) as a model for vascular dopamine receptors.

Komidori et al. (1992) recommended the isolated rat mesenteric vascular-intestinal loop preparation as an excellent model fort demonstrating resistance changes in isolated vascular beds while simultaneously measuring endogenous catecholamine overflow.

Pelissier et al. (1992) showed that perfusion with hypotonic solutions removed the endothelial layer in the isolated perfused mesenteric vascular bed of the rat, allowing the study of endothelial-dependent vascular responses.

Santiago et al. (1993) used the mesenteric vascular bed of the **cat** to study the inhibitory effects of the bradykinin receptor antagonist Hoe 140 on vascular responses to bradykinin.

The responses of adrenomedullin and adrenomedullin analogs in the mesenteric vascular bed of the cat were compared by Santiago et al. (1995).

Chu and Beilin (1994) studied the mesenteric vascular reactivity which is reduced in pregnant rats after application of bradykinin and the bradykinin receptor antagonist Hoe 140.

Mulavi and Halpern (1977), Qiu et al. (1995) studied the mechanical and contractile properties of *in situ* localized mesenteric arteries in normotensive and spontaneously hypertensive rats.

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A.8.2.6 Effect on pulmonary blood flow

PURPOSE AND RATIONALE

During controlled pulmonary blood flow, lobar arterial pressure can be measured in anesthetized cats (Lippton et al. 1984; Hyman et al. 1989; McMahon and Kadowitz 1993; deWitt et al. 1994).

PROCEDURE

Adult cats of either sex weighing 2.5 to 4.5 kg are sedated with ketamine hydrochloride (10–15 mg/kg i.m.) and anesthetized with pentobarbital sodium (30 mg/kg i.v.). The animals are fixed in supine position and supplemental doses of anesthetic are administered to maintain a uniform level of anesthesia. The trachea is intubated and the animals breath room air enriched with 95% O_2/5% CO_2. Systemic arterial pressure is measured from a catheter inserted into the aorta from a femoral artery, and intravenous injections are made from a catheter positioned in the inferior vena cava from a femoral vein.

For perfusion of the left lower lung lobe, a special designed 28-cm 6F triple-lumen balloon perfusion catheter (Arrow International, Reading, PA) is passed under fluoroscopic guidance from the left external jugular vein into the artery to the left lower lobe. The animal is heparinized by 1 000 IU/kg i.v., and the lobar artery is isolated by distension of the balloon cuff on the perfusion catheter. The lobe is then perfused by way of the catheter lumen beyond the balloon cuff, with blood withdrawn from a femoral artery with a perfusion pump. Lobar arterial pressure is measured from a second port 5 mm from the cuff on the perfusion catheter. The perfusion rate is adjusted so that lobar arterial perfusion pressure approximates mean pressure in the main pulmonary artery. Left atrial pressure is measured with a 6F double-lumen catheter passed transseptally into the vein draining the left lower lobe. The catheter tip is positioned so that the left atrial pressure port on the distal lumen is 1–2 cm into the lobar vein and the second catheter port is near the venoatrial junction.

Lobar arterial pressure can be elevated to an high steady state level by the administration of N^{ω}-nitro-L-arginine$^{\omega}$, followed by an intralobar infusion of the stable prostaglandin/endoperoxide analogue U-46619.

EVALUATION

Dose-response curves after administration of graded doses of drugs, e.g., decrease of lobar arterial pressure after various doses of bradykinin, are established. The effects of antagonists, e.g. HOE 140, can be studied.

MODIFICATIONS OF THE METHOD

Liu et al. (1992) used a blood-perfused rat lung preparation to study pulmonary vasoconstriction or endothelium-dependent relaxation.

Byron et al. (1986) studied the deposition and airway-to-perfusate transfer of disodium fluorescein from 3–4 μm solid aerosols in an isolated perfused lung preparation of rats.

Mor et al. (1990) determined angiotensin-converting enzyme activity in the isolated perfused **guinea pig** lung.

Franks et al. (1990) used in Beagle **dogs** a single breath technique employing freon-22 as the soluble marker gas simultaneously with measurement of aortic blood flow by an electromagnetic flowmeter.

Tanaka et al. (1992) measured lung water content in dogs with acute pulmonary hypertension induced by injection of glass beads.

Drake et al. (1978) studied filtration characteristics of the exchange vessels in isolated dog lung by calculating the volume conductance with use of different components of the weight-gain curve following changes in capillary pressure.

Heaton et al. (1995) studied the effects of human adrenomedullin on the pulmonary vascular bed of isolated, blood perfused rat lung.

DeWitt et al. (1994), Lippton et al. (1994) investigated the effects of adrenomedullin in the pulmonary and systemic vascular bed of the **cat**.

Nossaman et al. (1995) compared the effects of adrenomedullin, an adrenomedullin analog, and CGRP in the pulmonary vascular bed of the cat and the rat.

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A.8.2.7
Effect on contractile force of ischemic muscle

PURPOSE AND RATIONALE

Skeletal muscle is stimulated until it reaches its maximal force of contraction. This means that the muscle is forced to function at a level near exhaustion and has to use maximally the substrate supplied by the circulation. Then, the femoral artery is occluded, leading to underperfusion and a subsequent lack of substrate. As a consequence, the muscle's force of contraction decreases rapidly. Measuring the change in contractile force caused by drug administration reveals a drug's ability to restore ischemic muscle functions. In the following procedure, the drugs are tested for their effect on the force of contraction of the ischemic skeletal muscle.

PROCEDURE

Male Wistar rats weighing 400–450 g are anaesthetized by intraperitoneal administration of pentobarbital (Nembutal®) (35 mg/kg). A tracheal tube is placed to assist the rat's ventilation. The left carotid artery is cannulated for blood pressure recording and the left jugular vein is cannulated for the i.v. administration of test drugs. An incision is made to the skin of the right pelvic limb distal to the groin and the skeletal muscle is exposed down to the ankle. The skin is carefully trimmed away from the muscle to assure that contractions cannot be impaired by retraction of the skin. The major nerve supply is severed and a small length of the descending branch of the femoral artery is prepared free. The freely hanging muscle is attached to the force transducer (range 0–500 g, Z 6, Rhema, Germany) and a resting tension of 50 g is placed on the muscle. To prevent dehydration, the skin is left attached to the muscle and the muscle is kept moist by the continuous drip of a 0.9% NaCl-solution.

After these preparations, the rat is allowed to recover at least 30 min. Two needle electrodes are inserted into the muscle. Square impulses of 40 ms are generated with Stimulator 1 (Hugo Sachs Elektronik, Freiburg, Germany). The muscle is stimulated with a frequency of about 80 contractions per minute. The amplitude is increased gradually up to the muscle's maximal contractile force (usually between 2.0 and 3 mA). Following stabilization, the femoral artery is occluded with a clip for 5 min and subsequently reopened. After at least 15 min, test drugs are administered by intravenous infusion (0.075 ml/min) for 10 min. Five minutes after starting drug infusion, the artery is clamped again (for 5 min) while drug infusion is still going on. The force of contraction is continuously recorded. After declamping the artery, the rat is allowed to recover for at least 30 min before the whole procedure is repeated with another test drug. In this way, 3 different compounds can be tested in the same animal.

Standard compound:
- pentoxifylline

EVALUATION

The following parameters are measured:

- the percent inhibition of contractile force before drug administration (artery being clamped)
- the percent inhibition of contractile force after drug administration (artery being clamped)

The percent increase in contractile force after drug administration is calculated.

CRITICAL ASSESSMENT OF THE METHOD

An attempt is made to measure not only the effects of the drug on vasculature tonus but also on muscle metabolism.

MODIFICATIONS OF THE METHOD

Weselcouch and Demusz (1990) studied drug effects in the ischemic hindlimbs of **ferrets**. The hindlimb was stimulated to contract isometrically via supramaximal electrical stimulation of the sciatic nerve. Ischemia was induced by partial occlusion of the abdominal aorta. Pentoxifylline attenuated the loss of function in a dose-related manner.

Okyayuz-Baklouti et al. (1992) studied the functional, histomorphological and biochemical changes in atrophying skeletal muscle using a novel immobilization model in the rat.

Le Tallec et al. (1996) reported the effects of dimethylformamide on *in vivo* fatigue and metabolism in rat skeletal muscle measured by 31P nuclear magnetic resonance spectroscopy.

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A.8.2.8 Effect on perfusion of rabbit ear (Pissemski method)

PURPOSE AND RATIONALE

The procedure was described as early as 1914 by Pissemski, based on experiments of Krawkow (1913) in fish gills. It can be used to elucidate vasoactive properties (both vasoconstrictive and vasodilating) of compounds. The isolated rabbit's ear is used to determine the effect of test compounds on its perfusion rate. Administration of norfenephrine induces vasoconstriction leading to a decrease in perfusion rate. A compound with vasodilatory properties will inhibit the norfenephrine induced fall in perfusion rate whereas a vasoconstrictor will potentiate this effect.

PROCEDURE

A rabbit of either sex weighing 1.5–3 kg is sacrificed by CO_2 narcosis and its ears are severed immediately. The ear is placed on a glass disc, the posterior auricular artery is exposed and cannulated. The cannula is connected to a tubing with a T-branch allowing the infusion of different solutions. Ringer's solution, kept at room temperature, is infused under 40 cm water column pressure via the cannula. The perfusion flow volume is recorded using a time ordinate recorder and a CONDON tipper or a photoelectric drop counter.

Prior to drug administration, the pH of the Ringer solution (containing test compound) must be determined. If the pH is greater than 8.5 or smaller than 6.5, it should be adjusted by adding a diluted NaOH- or HCl-solution.

Testing for vasodilatory (norfenephrine antagonistic) activity

Norfenephrine is infused at a concentration of 0.5 µg/ml until the maximal contraction is reached. The test compound is prepared in Ringer's solution at a concentration of 100 µg/ml. A volume of 30 ml is infused via the cannula over a 15 min period under constant pressure. The change of perfusion rate is determined. If there is a positive response (increase in perfusion rate), the test may be repeated using lower concentrations. If there is a negative response (further decrease in perfusion rate), the compound can be tested for vasoconstrictive activity.

Testing for vasoconstrictive activity

This test is repeated as described above without administration of norfenephrine.

Standard compounds:

- as vasodilator
  - dihydralazine
  - theophylline
  - pentoxyfylline 100 µg/ml
- as vasoconstrictor
  - norfenephrine (Novadral®)

EVALUATION

Testing for vasodilatory activity

The perfusion rate of the ear vessel is determined during the course of the test:

R = perfusion rate of vehicle perfused vessel
RN = perfusion rate of norfenephrine constricted vessel
RNP = perfusion rate of norfenephrine constricted vessel following compound administration

The percent inhibition of norfenephrine induced decrease in perfusion pressure is calculated using the following formula:

$$\% \text{ inhibition} = \frac{(RN - RNP)}{R - RN} \times 100$$

Testing for vasoconstrictive activity

The normal perfusion rate (ear vessel perfused with Ringer's solution) is taken as 100%. The percent inhibition of perfusion rate following compound administration is determined.

MODIFICATIONS OF THE METHOD

Schlossmann (1927) used the isolated rabbit ear preparation according to Pissemski for determination of the adrenaline content in blood.

De la Lande and Rand (1965), de la Lande et al. (1967) described a method of perfusing the isolated central artery of the rabbit ear. Small segments of the artery, taken from the base of the ear, were perfused at a constant rate with Krebs solution at 37°C. To enable drugs to be applied either to the intima or the adventitia, the artery was double cannulated so that the intraluminal and extraluminal perfusion media did not mix. Constrictor responses were measured by the maximum rise in perfusion pressure.

Steinsland et al. (1973) studied the inhibition of adrenergic transmission by parasympathicomimetics in the isolated central ear artery of the rabbit. Perfusion was performed at a constant flow rate with Krebs' solution and perfusion pressure was recorded with a transducer.

Allen et al. (1973) incubated isolated segments of rabbit ear artery with (^{3}H)-(−)-noradrenaline and measured the amount of tritium released into the luminal perfusate and into the extraluminal superfusate.

Budai et al. (1990) used isolated proximal 3–4-cm segments of the rabbit ear artery or rat tail artery in a low volume perfusion-superfusion system for measurement of transmitter release from blood vessels *in vitro*.

Miyahara et al. (1993) used arterial rings rabbit ear arteries *in vitro* which were contracted by perivascular nerve stimulation, or 5×10^{-7} noradrenaline or high potassium (29.6 mM) solution. High doses of dexamethasone or clobetasol-17-propionate decreased the amplitude of contractions. Furthermore, the authors performed *in vivo* experiments in albino rabbits, whereby the fur was removed from the distal parts of the ear by applying a depilatory cream at least 24 h before the experiments. The apical regions of the ear were then stripped with adhesive tape 7 times to remove the keratinous epidermal layer. The rabbit was anesthetized and the experimental parts of the ear were placed under a high resolution magnifying camera and immobilized using bilayer adhesive tapes. The vascular reactions induced by topical application of corticosteroids were recorded chronically using video-tapes.

Aoki and Chiba (1993) described a method for separate intraluminal and extraluminal perfusion of the **basilar artery** in dogs. A polyethylene roof was designed to cover the canine basilary artery so that an extraluminal superfusion stream could pass over the artery that simultaneously received an intraluminal perfusion.

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A.8.2.9
Effect on venous tonus *in situ* in dogs

PURPOSE AND RATIONALE

Veins can be classified into two groups: those that respond and those that do not respond to epinephrine, acetylcholine and sympathetic nerve stimulation. As studies in dogs have shown (Rice et al. 1966) the reactive veins have a considerable amount of smooth muscle whereas the nonreactive ones lack any appreciable amount of smooth muscle fibres in the tunica media. A special preparation allows the registration of localized venous vasoconstriction.

PROCEDURE

Dogs weighing 20–30 kg are anesthetized with 35 mg/kg pentobarbital sodium i.v. The trachea is cannulated and the dog ventilated with a respiration pump. The femoral vein is cannulated for systemic injections. After administration of 5 mg/kg heparin sodium i.v., the saphenous vein and the femoral artery are cannulated. The venous cannula is placed approximately 1 cm distal to its junction with another vein. After the nonperfused branch of the junction is ligated, constant blood flow is maintained from the femoral artery by using a Sigmamotor® pump. The flow is adjusted so that a normal physiologic pressure in the vein is maintained. Perfusion pressure is measured between the pump and the vein so that any changes in pressure reflect changes in venous resistance. The peak changes in perfusion pressure are used to measure pressure changes from recorded data. The blood flow is maintained on a constant level. Therefore, changes in pressure must reflect changes in resistance. Pressure is recorded with a polygraph using an Statham pressure transducer (P23AA). In addition to recording perfusion pressure, venous pressure is measured at two additional points along the vein. In order to record venous pressure centrally from the site of perfusion, the shaft of a 27-gauge needle is placed into the end of a 10 cm piece of a thin Silastic tubing. At the other end, a 27 gauge needle is inserted and attached to a Statham pressure transducer (P23B). Pressure is recorded on a polygraph. One needle is inserted into the vein just proximal of the junction of the two veins. The second needle is placed into the vein so that the distance between the tip of the perfusion cannula and the first needle is the same as the distance between the two needles. In this way pressure decreases across the junction and an adjacent segment can be measured simultaneously. Injections of test compounds are made into the cannula between the pump and the saphenous vein. Changes in pressure measured by the three transducers are recorded. As standard, doses of 0.1–1.0 µg norepinephrine are injected.

EVALUATION

Responses to test drugs are measured in mm Hg and calculated as percentage of response to norepinephrine.

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A.8.3
Angiogenesis and anti-angiogenesis

A.8.3.1
General considerations

Regulation of new blood vessel formation, angiogenesis, is precisely programmed throughout the lifetime of vertebrates. Besides the role of angiogenesis in normal function, it is an essential component of disease processes, including tumor growth, rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman and Klagsbrun 1987; Klagsbrun and D'Amore 1991; Folkman and Shing 1992). Multiple factors that stimulate angiogenesis either directly or indirectly have been described, including the fibroblast growth factor family (Esch et al. 1985), vascular endothelial growth factor (Leung et al. 1990; Thomas 1996; Ferrara and Davis-Smyth 1997), epidermal growth factor (Gospadarowicz et al. 1979), transforming growth factor-α and -β (Schreiber et a 1986; Yang and Moses 1990), tumor necrosis factor-α (Leibovich et al. 1987), angiogenin (Fett et al. 1985), CYR61, a product of a growth factor-inducible immediate early gene (Babic et al. 1998) ect. The pharmacological inhibition of angiogenesis is of considerable interest in the development of new therapeutic modalities for the treatment of diseases such as diabetic retinopathy, atherosclerosis, hemangiomas, rheumatoid arthritis and cancer, in which pathological angiogenesis occurs (Ezekowitz et al. 1992; Folkman and Shing 1992; Fan and Brem 1992; O'Brien et al. 1994). Several natural inhibitors of angiogenesis were described, such as thrombospondin (Good et al. 1990), somatostatin (Barrie et al. (1993), angiostatin, isolated from a subclone of Lewis lung carcinoma, (O'Reilly et al. 1994, 1996), endostatin, a 20 kDa angiogenesis inhibitor from a murine hemangioendothelioma which is a C-terminal fragment of collagen XVIII (O'Reilly et al. 1997; Dhanabal et al. 1999), vasostatin (Pike et al. 1998).

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A.8.3.2 Endothelial cell proliferation

PURPOSE AND RATIONALE

Human umbilical vein endothelial cells (HUVEC) were used by various authors to study endothelial cell proliferation (Bussolino et al. 1992; Benelli et al. 1995; Danesi et al. 1997; Hu 1998; Iurlaro et al. 1998; Vacca et al. 1999; Xin et al. 1999).

PROCEDURE

The HUV-EC-C human endothelial cells (American Type Culture Collection, Rockville, MD) are cultured at 37 °C and 5% CO_2 in 90% Ham's F12K, 10% fetal bovine serum, 30 µg/ml endothelial cell growth factor, 100 µg/ml heparin, and 4 mM L-glutamine. The effect of test compound on HUV-EC-C cell proliferation is evaluated on 3×10^3 cells/well in 24-well plates. After 24 h, the test compound in various concentrations of the vehicle are added, and plates are incubated for 72 h. Cells are then harvested with trypsin/EDTA and counted by an hemocytometer.

EVALUATION

Results are expressed as number of cells in vehicle and compound-treated cultures and are the mean of three separate experiments ±SE.

MODIFICATIONS OF THE METHOD

In addition to human umbilical veil endothelial cells, Pike et al. (1998) used fetal bovine heart endothelial cells and measured DNA synthesis by [^{3}H]thymidine deoxyribose uptake.

Oikawa et al. (1991) used vascular cells from bovine carotid arteries and tested cell proliferation in a collagen gel and cell migration in a Boyden chamber.

Bovine capillary endothelial cells were used by Folkman et al. (1979), Clapp et al. (1993), O'Reilly et al. (1997), Cao et al. (1999).

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A.8.3.3 Chorioallantoic membrane assay

PURPOSE AND RATIONALE

The chick chorioallantoic membrane assay, originally described by Auerbach et al. (1974), has been used with some modifications by several authors to test angiogenesis and inhibition of angiogenesis, e.g., by Taylor and Folkman (1982), Crum et al. (1985), Vu et al. (1985), McNatt et al. (1992, 1999), Barrie et al. (1993), Clapp et al. (1993), Gagliardi and Collins (1993), Benelli et al. (1995), Ribatti et al. (1995), Klauber et al. (1996), Oikawa and Shimamura (1996), Danesi et al. (1997), O'Reilly et al. (1997), Iurlaro et al. (1998), Cao et al. (1999), Vacca et al. (1999).

PROCEDURE

Fertilized White Leghorn chicken eggs are incubated at 37 °C at constant humidity. On incubation day 3, a square window is opened in the shell and 2 to 3 ml of albumen is removed to allow detachment of the developing chorioallantoic membrane (CAM). The window is sealed with a glass and the eggs are returned to the incubator. On day 8, 1 mm^3 gelatin sponges loaded with 3 µl phosphate-buffered saline alone as the negative control or containing 3 µg (1 mg/ml) of the angiogenic recombinant basic fibroblast growth factor alone as positive control, or together with various doses of test compound, are implanted on top of the CAM. The sponge traps the sample and allows slow release of the product. CAM are examined daily until day 12, when the angiogenic response peaks. On day 12, blood vessels entering the sponge within the focal plane of the CAM are recognized microscopically, counted by two observers in a double-blind fashion under a Zeiss SR stereomicroscope, and photographed in ovo with the MC63 Camera system (Zeiss, Oberkochen, Germany). To better highlight vessels, the CAM are injected into a large allantoic vein with India ink solution, fixed in Serra's fluid, dehydrated in graded ethanols, and rendered transparent in methylbenzoate. On day 12, after microscopic counting, the embryos and their membranes are fixed in ovo in Bouin's fluid. The sponges and the underlying and immediately adjacent CAM portions are removed, embedded in paraffin, sectioned at 8 µm along a plane parallel to the CAM surface, and stained with a 0.5% aqueous solution of toluidine blue.

EVALUATION

Angiogenesis is measured by a planimetric point count method (Ribatti et al. 1999): four to six 250× magnification fields covering almost the whole of every third section within 30 serial slides of each sponge per sample are analyzed within a superimposed 144 intersection point square reticulum of 0.125 mm^2. Only transversely sectioned microvessels, ie, capillaries and venules with or without a 3 to 10 µm lumen occupying the intersection points, are counted and calculated as the mean ±1 SD per section, per CAM, and groups of CAM. Statistical significance of differences is cal-

culated by comparing the data from each experiment to their controls using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Oh et al. (1997) studied the lymphatics of differentiated avian chorioallantoic membrane using microinjection of Mercox resin, semi- and ultrathin sectioning, immunohistochemical detection of fibronectin and α-smooth muscle actin, and *in situ* hybridization with vascular endothelial growth factor VEGFR-2 and VEGFR-3 probes.

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A.8.3.4 Cornea neovascularization

PURPOSE AND RATIONALE

Neovascularization of the rabbit cornea has been used by several authors to study inhibition of angiogenesis (Gimbrone et al. 1974; Crum et al. 1985; BenEzra et al. 1987, 1997; Klauber et al. 1996; McNatt et al. 1999; Joussen et al. 1999).

PROCEDURE

New Zealand White rabbits are anesthetized for surgery and quantification of newly developed blood vessels with 5 mg/kg xylazine hydrochloride and 35 mg/kg ketamine hydrochloride i.m. Corneal blood vessels are induced by basic fibroblast growth factor which is applied in carrier pellets. These pellets are produced by dispersing 50 µl of 2% methylcellulose containing 500 ng human recombinant basic fibroblast growth factor diluted in 10 µl phosphate-buffered saline in plastic rods with a diameter of 4 mm. Dried pellets are folded twice and implanted intrastromally in the 12 o'clock position into a corneal tunnel. This tunnel is created by a central cut of approximately 50% depth and extended into the peripheral cornea to a point 2.0 mm away from the limbus. Following implantation, the central entrance of the tunnel is closed with a single 10-0 nylon suture in order to ensure that the tear film does not dissolve the pellet and uncontrolled liberation of the growth factor is prevented.

The test substance is dissolved in a viscous gel containing 0.002% polyacrylic acid, 0.04% sorbitol, and 0.001% cetrimide in a watery base. The eyes are treated once daily with 0.1 ml of this gel which is applied in the lower conjunctiva sac. The eyes are closed for sev-

eral seconds to avoid loss of the substance. Each animal's contralateral eye receives gel without test substance and serves as control. Control animals receive the viscous gel without test substance.

Animals are observed daily under an operating microscope, and vascular growth is documented on days 6, 9, 12, and 16 after surgery. The number of blood vessels, their length and the dimension of the vascularized area are quantified with a caliper under the operating microscope as well as on standardized photographs. On every observation day corneas are stained with fluorescein in order to show epithelial irregularities due to the topical treatment.

EVALUATION

Differences between treated eyes and controls are tested for significance using unpaired Student's *t*-test.

MODIFICATIONS OF THE METHOD

Damms et al. (1997) characterized the neovascularization that follows the intracorneal injection of bovine albumin in rabbits as a model of angiogenesis. New Zealand white rabbits received intracorneal injections of phosphate buffered saline with and without various amounts of bovine albumin. The rabbits were co-sensitized or pre-sensitized by intramuscular bovine albumin. The corneal response was quantified by ranking photographs taken periodically after the injection.

Babic et al. (1998) tested an angiogenesis promotor in the corneal pocket angiogenesis assay in **rats**.

Xin et al. (1999) studied inhibitors of angiogenesis in the corneal angiogenesis assay in rats. A 1.5 mm incision was made approximately 1 mm from the center of the cornea in anesthetized Sprague Dawley rats. Using a curved spatula, the incision was bluntly dissected through the stroma toward the outer canthus of the eye. A hydron pellet (2×20 mm) containing 200 ng vascular endothelial growth factor and 100 ng sucralfrate was inserted into the base of the pocket.

Foschi et al. (1994), Benelli et al. (1995), Danesi et al. (1997) studied neovascularization of rat cornea induced by **chemical injury**. Both eyes of ether-anesthetized rats were cauterized by applying a $AgNO_3$/KNO_3 (1:1, w/w) applicator to the surface of the cornea eccentrically at a point approximately 2 mm from the corneoscleral limbus. Rats were treated 4 times daily for 6 days with eye drops. The eyes were examined by slit-lamp microscopy daily for 6 days to the evaluate the growth of the vessels. On the 6^{th} day after cauterization, the rats were anesthetized and the upper body perfused through a cannula inserted in the ascending aorta with Ringer's solution until the normal pink color of the fundi disappeared and then with a mixture of 10% India ink/6% gelatin in Ringer's solution. The eyes were enucleated and placed in 4% formaldehyde. The cornea and a 1 mm rim of adjacent scleral tissue were dissected from the rest of the globe and three full thickness peripheral radial cuts were made to allow flattening of the cornea. The corneas were then placed on a glass slide in mounting media, magnified, and photographed. The area occupied by blood vessels was calculated and the area vascularization of drug treated animals was compared to that of control rats.

Kenyon et al. (1996), Cao et al. (1999) performed the corneal micropocket assay in **mice**.

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A.8.3.5 Rat subcutaneous air sac model

PURPOSE AND RATIONALE

Lichtenberg et al. (1997, 1999) recommended the subcutaneous air sac model in rats as a simple method for *in vivo* screening of antiangiogenesis. Subcutaneous injection of air in the rat results in the formation of an air pouch. If air pouches are kept inflated by repeated injections of air the develop a structure of synovial lining (Edwards et al. 1981). The subcutaneous air pouch appears more like a bursal cavity than a synovial joint (Kowanko et al. 1986) and after 8–10 days the cells of the air sac appear as a transparent membrane on which the formation of new vessels can be studied.

PROCEDURE

Under anesthesia 10–15 ml of air is introduced dorsally to female Sprague Dawley rats weighing 150–180 g by subcutaneous injections using a 25 gauge needle to produce an air sac located approximately 4–5 cm behind the head of the animal. The air sacs are re-inflated every forth day. The wall of the air sac becomes progressively thicker with time and after approximately 10 days a sufficient lining of cells has been established. For sponge implantation, the animals are anesthetized again. A 1.5 cm incision is made through the clipped skin covering the air sac and blunt dissection is used to open a 2 cm deep cavity towards the cranial base of the air sac by careful separation of the skin from the membrane. A cellulose implant (Spontex® sponge) with a diameter of 8 mm is carefully pressed into the cavity of the membrane away from the incision site and the incision closed by sutures. The animals are treated for 10 days with various doses of test compound in a volume of 10 ml/kg or vehicle. The subcutaneous injection is made under light CO_2/O_2 anesthesia into the hind leg 5–7 cm away from the air sac. This injection site is chosen to eliminate any risk of inducing irritative side-effects on the membrane. After 10 days treatment, the animals are sacrificed after having received 20 min before an injection of 1 μCi of ^{125}I-labelled immunoglobulin via the tail vein. The overlying skin of the air sac is removed to expose the transparent membrane. The extent of vascular proliferation is scored *in situ*:

1+: slight background vascularization;
2+: few new vessels reach the sponge;
3+: many new vessels reach and penetrate the implant;
4+: very intense formation of new vessels which reach and penetrate the implant.

Following *in situ* scoring, the implant and the membrane from each animal are placed in the same plastic vial containing 10% formalin and the radioactivity is measured in a γ-counter. The implants are examined microscopically after staining with haematoxylin and eosin.

EVALUATION

The extent of vascular proliferation scored *in situ* is compared between vehicle and treated animals by the Wilcoxon test. The angiogenic response measured by ^{125}I-activity in cpm is subjected to analysis of variance followed by Dunnett's *t*-test to compare each dose with the vehicle. The cpm's are log-transformed to obtain variance homogeneity. The correlation between *in situ* scores and cpm is estimated by Spearman's rank correlation coefficient after ranking cpm values.

MODIFICATIONS OF THE METHOD

In a further study, Lichtenberg et al. (1999) inoculated vascular endothelial growth factor producing tumor cells subcutaneously directly on the membrane, and the formation of vessels was measured 8 days later. Furthermore, slow-release pellets containing angiogenic factors, basic fibroblast growth factor or vascular endothelial growth factor, were implanted on the subcutaneous membrane.

Nakamura et al. (1999) studied suppression of angiogenesis induced by S-180 mouse tumor cells in the dorsal air sac assay in **mice**.

Funahashi et al. (1999) developed a mouse dorsal air sac model for quantifying *in vivo* tumor-induced angiogenesis which is determined by measuring the blood volume in an area of skin held in contact with a tumor cell-containing chamber, using ^{51}Cr-labeled red blood cells.

Schreiber et al. (1986) described the **hamster cheek pouch assay** for testing angiogenic/antiangiogenic activity.

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A.8.3.6
Mesenteric window angiogenesis model

PURPOSE AND RATIONALE

Norrby et al. (1986, 1990, 1995) described the mesenteric window assay in rats for quantitative measurement of induction and inhibition of angiogenesis. The tissue being used is the membranous, 'window'-like parts of the mesentery which is normally vascularized and appears to lack significant physiologic angiogenesis. Since the mesenteric window natively measures only 5–10 μm in thickness, the vasculature is virtually two-dimensional. Due to the structural and metabolic simplicity of the test tissue, the mesenteric window microvasculature is regarded as an ideal test system for establishing the functional influences of defined factors (Zweifach 1973).

PROCEDURE

Angiogenesis is induced by i.p. injection of the mast-cell secretagogue compound 48/80 twice daily for 4.5 days to male Sprague Dawley rats weighing about 225 g. Test compounds or saline are injected s.c. 1 h before each injection of compound 48/80.

Angiogenesis is quantified by microscopically counting the number of vessel profiles per unit length of the mesenteric window in 4 microtome sections per specimen, cut perpendicularly to the surface, from the central part of the window. This reflects the degree of branching, the degree of tortuosity and the degree of spatial expansion of the vasculature. Four specimens per animal are analyzed.

Four mesenteric window specimens are spread, fixed on objective slides and stained with toluidine blue to measure the relative vascularized area. Three randomly selected vascular view fields per mesenteric-window spread are analyzed for microvascular length per unit area of vascularized tissue. The total microvascular length is computed from the vascularized area of each animal multiplied by the mean microvascular length for the corresponding treatment group.

EVALUATION

The non-parametric two-tailed Mann-Whitney U rank sum test for unpaired observations is used for statistical analysis.

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Chapter B
Activity on blood constituents[1]

[1] Contributed by M. Just and V. Laux.

B.1 *In vitro* tests

B.1.1 Blood coagulation tests

PURPOSE AND RATIONALE

The coagulation cascade consists of a complex network of interactions resulting in thrombin-mediated cleavage of fibrinogen to fibrin which is one major component of a thrombus. The coagulation cascade can be initiated either by the "exogenous pathway", the release of thromboplastin (tissue factor) leading to activation of factor VII to the tissue factor/factor VIIa complex, or by the "endogenous pathway", so-called contact activation leading via factors XII, XI and IX to the assembly of the tenase complex consisting of activated factors VIII and IX and Ca^{2+} on a phospholipid surface. Both complexes can activate factor X which induces the formation of the prothrombinase complex consisting of factor X_a, factor Va and Ca^{2+} on a phospholipid surface. The latter leads to the activation of thrombin which in turn cleaves fibrinogen to fibrin. The three coagulation tests (PT, APTT and TT) allow to differentiate between effects on the exogenous or endogenous pathway or on fibrin formation. The influence of compounds on the plasmatic blood coagulation is determined by measuring the coagulation parameters prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) *ex vivo*.

PROCEDURE

Male Sprague-Dawley rats weighing 200–220 g receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time, they are anesthetized by intravenous injection of 60 mg/kg sodium pentobarbital. The caudal caval vein is exposed by a mid-line incision and 1.8 ml blood are collected into a plastic syringe containing 0.2 ml 100 mM citrate buffer pH 4.5 (Behring Werke, Marburg). The sample is immediately agitated and centrifuged in a plastic tube at 1 500 *g* for 10 min. Plasma is transferred to another plastic tube and the coagulation tests for the determination of thrombin time (TT), thromboplastin time (PT) and activated partial thromboplastin time (APTT) are performed within 3 h.

In general, citrated plasma is coagulated by the addition of the respective compounds (see below) and the time to clot formation is determined in the coagulometer (= coagulation time).

Prothrombin Time (PT). An aliquot of 0.1 ml of citrated plasma is incubated for 1 min at 37 °C. Then 0.2 ml of human thromboplastin (Thromborel®, Behring Werke, Marburg) is added and the coagulometer (Schnittger + Gross coagulometer, Amelung, Brake) is started. The time to clot formation is determined. The PT measures effects on the exogenous pathway of coagulation.

Activated Partial Thromboplastin Time (APTT). To 0.1 ml of citrated plasma 0.1 ml of human placenta lipid extract (Pathrombin®, Behring Werke, Marburg) is added and the mixture is incubated for 2 min at 37 °C. The coagulation process is initiated by the addition of 0.1 ml 25 mM calcium chloride when the coagulometer is started and the time to clot formation is determined. The APTT measures effects on the endogenous pathway of coagulation.

Thrombin Time (TT). To 0.1 ml of citrated plasma 0.1 ml of diethylbarbiturate-citrate buffer, pH 7.6 (Behring Werke Marburg) is added and the mixture is incubated for 1 min at 37 °C. Then 0.1 ml of bovine test-thrombin (30 IU/ml, Behring Werke Marburg) is added and the coagulometer is started. The time to clot formation is determined. The TT measures effects on fibrin formation.

EVALUATION

Mean values of TT, PT and PTT are calculated in dosage groups and vehicle controls. Statistical evaluation is performed by means of the unpaired Student's *t*-test.

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B.1.2 Thrombelastography

PURPOSE AND RATIONALE

Thrombelastography (TEG) was developed first by Hartert (1948). The thrombelastograph (Haemoscope Corp., Skokie, Illinois, USA) is a device that provides a continuous recording of the process of blood coagulation and subsequent clot retraction. The blood samples are transferred to cuvettes and maintained at 37 °C. The cuvettes are set in motion around their vertical axes. Originally, a torsion-wire suspended mirror in the plasma remains immobile as long as the plasma is fluid. The cuvette and the mirror become dynamically related as fibrin forms, resulting in transmission of cuvette motion to the mirror. The mirror then oscillates with an amplitude governed by the specific mechanical properties of the clot and reflects its light to a thermopaper. The modern thrombelastograph transfers the analogous recording to a digital signal which is evaluated by a computer program.

PROCEDURE

Thrombelastography can be performed in either whole blood, or in citrated platelet rich or platelet poor plasma after recalcification. Blood samples are obtained from Beagle dogs, weighing 12–20 kg, from rabbits, weighing 1.7–2.5 kg or from Wistar rats weighing 150–300 g or from humans. The test subjects receive the compound by intravenous, subcutaneous or by oral administration. Ten or 20 min post dosing (i.v., s.c. ad-ministration) or 60, 90 or 180 min post dosing (oral administration) blood is collected. The blood samples are mixed with 3.8% trisodium citrate solution (one part citrate solution to 9 parts blood) as anticoaglant. The citrated whole blood is recalcified by adding 0.4 ml isotonic calcium chloride solution. An aliquot of 0.36 ml of the re-calcified whole blood is transferred to the pre-warmed cup of the thrombelastograph.After the apparatus has been correctly adjusted and the samples sealed with liquid paraffin to prevent drying, the time for the whole procedure is noted. The thrombelastogram is recorded for 2 h.

CRITICAL ASSESSMENT OF THE METHOD

Zuckerman et al. (1981) compared thrombelastography with other common coagulation tests (fibrinogen, prothrombin time, activated thromboplastin time, platelet count and fibrin split products) and found that there is a strong relationship between the thrombelastographic variables and these common laboratory tests. Moreover, TEG has an increased sensitivity for detecting blood clotting anomalies, it contains additional information on the hemostatic process. This is due to (1) the fact that most laboratory measurements end with the formation of the first fibrin strands while TEG measures the coagulation process on whole blood from initiation of clotting to the final stages of clot lysis and retraction, (2) the possibility of TEG to use whole non-anticoagulated blood without influence of citrate or other anticoagulants.

EVALUATION

The following measurements are the standard variables of TEG:

- **Reaction time (*r*):** the time from sample placement in the cup until onset of clotting (defined as amplitude of 1 mm). This represents the rate of initial fibrin formation.
- **Clot formation time (*k*):** the difference from the 1 mm *r* to 20 mm amplitude. *k* represents the time taken for a fixed degree of viscoelasticity achieved by the forming clot, caused by fibrin build up and cross linking.
- **Alpha angle (α°):** angle formed by the slope of the TEG tracing from the *r* to *k* value. It denotes speed at which solid clot forms.
- **Maximum amplitude (*MA*):** greatest amplitude on the TEG trace. *MA* represents the absolute strength of the fibrin clot and is a direct function of the maximum dynamic strength of fibrin and platelets.
- **Clot strength (*G* in dynes per square centimeter):** defined by $G = (5\,000\,MA) / (96 - MA)$. In a tissue factor-modified TEG (Khurana et al. 1997), clot strength is clearly a function of platelet concentration.
- **Lysis 30, Lysis 60 (*Ly30*, *Ly60*):** Reduction of amplitude relative to maximum amplitude at 30 and 60 min after time of maximum amplitude. These parameters represent the influence of clot retraction and fibrinolysis.

MODIFICATIONS OF THE METHOD

Bhargava et al. (1980) compared the anticoagulant effect of a new potent heparin preparation with a commercially available heparin by thrombelastography *in vitro* using citrated dog and human blood. Barabas et al. (1993) used fibrin plate assay and thrombelastography to assess the antifibrinolytic effects of synthetic thrombin inhibitors. Scherer et al. (1995) described a short-time, endotoxin-induced rabbit model of hypercoagulability for the study of the coagulation cascade and the therapeutic effects of coagulation inhibitors using various parameters including thrombelastography.

Khurana et al. (1997) introduced tissue factor-modified TEG to study platelet glycoprotein IIb/IIIa function and to establish a quantitative assay of platelet function. With this modification, Mousa et al. (2000) found two classes of glycoprotein IIb/IIIa antagonists, one with high binding affinity for resting and activated platelets and slow platelet dissociation rates (class I) demonstrating potent inhibition of platelet function, in contrast to those with fast platelet dissociation rates (class II).

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B.1.3 Chandler loop

PURPOSE AND RATIONALE

The Chandler loop technique allows to produce *in vitro* thrombi in a moving column of blood (Chandler 1958). The thrombi generated in the Chandler device show morphology very similar to human thrombi formed *in vivo* (Robbie et al. 1997) with platelet-rich upstream sections ("white heads") that are relatively resistant to t-PA-mediated thrombolysis in contrast to the red blood-cell-rich downstream parts ("red tails") (Stringer et al. 1994).

PROCEDURE

One millimeter of non-anticoagulated whole blood is drawn directly into a polyvinyl tube with a length of 25 cm and an internal diameter of 0.375 cm (1 mm = 9.9 cm tubing). The two ends of the tube are then brought together and closed by an outside plastic collar. The circular tube is placed and centered on a turntable, tilted to an angle of 23 degrees, and rotated at 17 rpm. At the moment the developing thrombus inside the tube becomes large enough to occlude the lumen, the blood column becomes static and moves around in the direction of rotation of the tube.

EVALUATION

Time to occlusion of the tube by the thrombus establishes a definite end point in this system.

MODIFICATIONS OF THE METHOD

Stringer et al. (1994) used this method to determine the influence of an anti-PAI-1 antibody (CLB-2C8) on the t-PA induced lysis of Chandler thrombi *in vitro*. They used citrated blood and supplemented it with 5.8 µM [^{125}I]-labelled fibrinogen prior to recalcification. After generation in the Chandler loop, the thrombi were washed with isotonic saline and then cut transversally into an upstream (head) and a downstream part (tail). The radioactivity of both parts was determined in a gamma counter (pre-value). The head and the tail were then subjected to thrombolysis by adding 300 µl phosphate-buffered saline containing plasminogen (2 µM) and t-PA (0.9 nM). During the observation time of 240 min aliquots of 10 µl were taken at 30, 60, 120, 180 and 240 min, and the radioactivity was determined. The relation of the measured radioactivity to the pre-value was expressed as percentage of clot lysis.

Van Giezen et al. (1998) used this method to differentiate the effect of an anti-PAI-1 polyclonal antibody (PRAP-1) on human or rat thrombi.

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B.1.4 Platelet aggregation and deaggregation in platelet rich plasma or washed platelets (BORN method)

PURPOSE AND RATIONALE

Platelets play a crucial role in primary hemostasis by forming hemostatic plugs at sites of vascular injury. Moreover, they contribute to intravascular thrombus formation mostly upon rupture of an atherosclerotic plaque. The contact of unactivated platelets to exposed subendothelial tissue leads to adhesion via two main mechanisms: binding of subendothelial von Willebrand factor (vWF) to the platelet GPIb-IX-V-complex at high shear rates and binding of collagen to two receptors, integrin $\alpha 2\beta 1$ and GPVI. Platelet adhesion initiates the reactions of shape change, secretion, and activation of GPIIb-IIIa-ligand binding sites. These reactions result in the formation of platelet aggregates. Activation of GPIIb-IIIa is also achieved through signaling by a number of agonists that bind to G-protein-coupled receptors. Consequently, for the measurement of platelet aggregation platelets are activated by the addition of one of the following agonists to platelet rich plasma (PRP) or washed platelets: ADP, arachidonic acid (forming thromboxane A2) or U 46619, collagen, thrombin or TRAP, serotonin, epinephrine, PAF. The formation of platelet aggregates with stirring leads to changes in optical density which are monitored photometrically usually for 4 min The test has been developed originally by Born (1962) and is used to evaluate quantitatively the effect of compounds on induced platelet aggregation *in vitro* or *ex vivo*. For *in vitro* studies, human PRP is preferred.

PROCEDURE

Materials and solutions

Anticoagulating substances:		
hirudin (Sigma)	200	µg/ml
trisodium citrate	0.11	M
ACD solution		
citric acid	38	mM
sodium citrate	75	mM
glucose	124	mM
Platelet aggregating substances: (final concentrations in the test)		
ADP: for reversible or biphasic aggregation	0.1–5	µM
ADP: for irreversible aggregation (Sigma)	3–6	µM
Sodium arachidonate (Biodata)	0.33	mM
Calcium ionophore A 23187 (Calbiochem)	10	µM
Collagen (Hormonchemie)	3	µg/ml
PAF-acether (C 16-PAF, Bachem)	0.1	µM
Thrombin (Sigma)	0.02–0.05	IU/ml
TRAP (SFLLRNP, Bachem)	10	µM
U 46619 (ICN)	1	µM
Ristocetin	1	mg/ml
GPRP (fibrin antipolymerant, Bachem)	0.5	mM
4-channel aggregometer (PAP 4, Bio Data)		

The test is carried out either *ex vivo* or *in vitro*.

For *ex vivo*-assays, mice, rats, or guinea pigs from either sex receive the test compound or the vehicle (for controls) by oral, intraperitoneal or intravenous administration. At the end of the absorption time, blood is collected by caval vein-puncture under pentobarbital sodium anesthesia and xylazine (8 mg/kg i.m.) premedication.

From rabbits (Chinchilla strain, weighing 3 kg), blood is withdrawn by cardiopuncture under xylazine (20 mg/kg i.m.) sedation. The first blood sample (control) is collected before administration of the test compound, the second sample at the end of the absorption time of the test agent.

For *in vitro*-assays, human blood is collected from the antecubital vein of adult volunteers, who had not received any medication for the last two weeks.

Preparation of PRP, PPP, and WP

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Freshly collected venous blood is anticoagulated with hirudin (1 volume + 9 volumes of animal blood) or ACD solution (1 volume + 9 volumes of human blood) and centrifuged at 170 *g* for 15 min to obtain platelet rich plasma (PRP). The PRP-supernatant is carefully removed, and the rest is further centrifuged at 1 500 *g* for 10 min to obtain platelet poor plasma (PPP). PRP is diluted with PPP to a platelet count of 3×10^8/ml before use in the aggregation assays. To obtain washed platelets (WP), 8.5 volumes of human blood are collected into 1.5 volumes of ACD and centrifuged as for PRP. PRP is acidified to a pH of 6.5 by addition of approximately 1 ml ACD to 10 ml PRP. Acidified PRP is centrifuged for 20 min at 430 *g*. The pellet is resuspended in the original volume with Tyrode's solution (mM: NaCl 120, KCl 2.6, $NaHCO_3$ 12, NaH_2PO_4 0.39, HEPES 10, glucose 5.5; albumin 0.35%) and set to platelet count of 3×10^8/ml.

EXPERIMENTAL COURSE

Studies should be completed within 3 h after blood withdrawal.

For *ex vivo*-assays, duplicate samples of 320 µl PRP from drug-treated and vehicle control subjects (for rabbits: control samples before drug administration) are inserted into the aggregometer at 37 °C under continuous magnetic stirring at 1 000 rpm. After the addition of 40 µl physiological saline and 40 µl aggregating agent, changes in optical density are monitored continuously at 697 nm.

For *in vitro*-assays, 40 µl of the test solution are added to samples of 320 µl PRP or WP from untreated subjects. The samples are inserted into the aggregometer and incubated at 37 °C for 2 min under continuous magnetic stirring at 1 000 rpm. After the addition of 40 µl aggregating agent, changes in optical density are monitored continuously at 697 nm either for 4 min or until constant values for aggregation are achieved. In cases of thrombin activation of PRP, GPRP is added in order to avoid fibrin formation.

In order to measure deaggregation, experimental compounds are added to stimulated PRP at 70 or 100% of control aggregation and monitoring is performed for further 10 min. Deaggregation is measured by the decrease of light transmission (see Haskel et al. 1989).

EVALUATION

The transmission maximum serves as a scale for platelet aggregation (0% = transmission of PRP, 100% = transmission of PPP).

For *in vitro*-assays:

1. Percent inhibition of platelet aggregation is determined in concentration groups relative to vehicle controls.

Statistical significance is evaluated by means of the unpaired Student's *t*-test.

2. IC_{50} values are determined from the non-linear curve fitting of concentration-effect relationships. IC_{50} is defined as the concentration of test drug for half maximal inhibition of aggregation.
3. Percent deaggregation is determined at 10 min after addition of compound; IC_{50} is calculated from the concentration-effect relationship.

For *ex vivo*-assays:

1. Mean values for aggregation in dosage groups are compared to the vehicle control groups (for rabbits: control values before drug administration).
   Statistical significance is evaluated by means of the Student's *t*-test (paired for rabbits; unpaired for others).
2. ED_{50} values are determined from the dose-response curves. ED_{50} is defined as the dose of drug leading to 50% inhibition of aggregation in the animals.

CRITICAL ASSESSMENT OF THE METHOD

The assay, introduced by Born (1962), has become a standard method in clinical diagnosis of platelet function disorders and of aspirin-intake. Furthermore, the method is used in the discovery of antiplatelet drugs with the advantage of rapid measurement of a functional parameter in intact human platelets

MODIFICATIONS OF THE METHOD

Several authors described modifications of the assay procedure. Breddin (1975) described spontaneous aggregation of platelets from vascular patients in a rotating cuvette. Klose et al. (1975) measured platelet aggregation under laminar flow conditions using a thermostated cone-plate streaming chamber in which shear rates are continuously augmented and platelet aggregation is measured from light transmission through a transilluminating system. Marguerie et al. (1979, 1980) developed a method measuring two phases of platelet aggregation after gel filtration of a platelet suspension (see below). Lumley and Humphrey (1981) described a method to measure platelet aggregation in whole blood (see below). Fratantoni et al. (1990) performed aggregation measurements using a microtiter plate reader with specific modification of the agitation of samples. Comparison of the 96well microtiterplate method with conventional aggregometry showed similar dose-response curves for thrombin, ADP, and arachidonic acid.

Ammit and O'Neil (1991) used a quantitative bioassay of platelet aggregation for rapid and selective measurement of platelet-activating factor.

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B.1.5 Platelet aggregation after gel filtration

PURPOSE AND RATIONALE

Triggering of platelet activation by low concentrations of ADP, epinephrine or serotonin – so-called weak platelet agonists – in plasma- and fibrinogen-free platelet suspensions does not result in platelet aggregation unless exogenous fibrinogen is added. As opposed to this, platelet aggregation induced by thrombin, collagen or prostaglandin-endoperoxide – so-called strong agonists – is independent of exogenous fibrinogen because these substances lead to the secretion of intracellular platelet ADP and fibrinogen. Studies of platelet aggregation in gel-filtered platelets are performed in cases where the adhesive ligand fibrinogen or vWF is needed in a defined concentration or where plasma proteins could negatively interfere with the effect of compounds. The assay is mostly used to evaluate the influence of compounds on platelet GPIIb-IIIa or other integrins or on GPIb-IX-V.

PROCEDURE

Materials and solutions

Acid-citrate-dextrose (ACD) solution:		
citric acid	0.8	%
sodium citrate	2.2	%
glucose	2.45	%
hirudin	0.6	U/ml
Tyrode's solution:		
NaCl	137	mM
KCl	2.7	mM
$MgCl_2$	5.5	mM
NaH_2PO_4	3.0	mM
HEPES	3.5	mM
glucose	5.5	mM
albumin	0.2	%
hirudin	0.06	U/ml
apyrase	40	µg/ml
pH	7.2	
Epinephrine (different concentrations; µM range)		
ADP	10	µM
Thrombin	0.02–0.05	U/ml
$CaCl_2$	0.5	mM
Fibrinogen (American Diagnostica)	1	mg/ml
von Willebrand factor	10	µg/ml
Sepharose CL 2B (Pharmacia)		
Acrylic glass column (Reichelt Chemietechnik, 3 cm inner diameter, 18 cm length)		
Aggregometer (PAP 4, Biodata)		

Preparation of gel filtered platelets

The entire procedure is performed in plastic (polystyrene) tubes at room temperature according to Marguerie et al.(1979).

Blood is drawn from healthy adult volunteers, who had no medication for the last two weeks. Venous blood (8.4 ml) is collected into 1.4 ml ACD-solution and centrifuged for 10 min at 120 *g*. The platelet rich plasma (PRP) is carefully removed, the pH adjusted to 6.5 with ACD-solution and centrifuged at 285 *g* for 20 min. The resulting pellet is resuspended in Tyrode's buffer (approx. 500 µl buffer/10 ml PRP). The platelet suspension is applied immediately to a Sepharose CL 2B column; equilibration and elution at 2 ml/min flow rate is done with Tyrode's buffer without hirudin and apyrase. Platelets are recovered in the void volume. Final platelet suspension is adjusted to 4×10^8/ml. Gel filtered platelets (GFP) are kept at room temperature for 1 h until the test is started.

Experimental course

For the aggregation studies, GFP in Tyrode's buffer is incubated with $CaCl_2$ (final concentration 0.5 mM) with or without fibrinogen (final conc. 1 mg/ml) in polystyrene tubes. After 1 min, 20 µl of the test compound or the vehicle (controls) are added and the samples are incubated for another 2 min. After the addition of 20 µl platelet agonist, changes in light transmission are recorded. The whole procedure is done under continuous magnetic stirring at 37 °C (1 000 rpm) in the aggregometer. Samples with added $CaCl_2$ but without fibrinogen identify proper exclusion of plasma proteins if neither spontaneous aggregation occurs nor aggregation in the presence of weak agonists. Full aggregatory response of GFP to 10 µM ADP shows intact platelets (with only minor pre-activation with gel-filtration).

EVALUATION

The transmission maximum serves as a scale for platelet aggregation. Each test compound is assayed with at least two different donor-GFP's; in the case of an anti-aggregating effect, the test is performed with 4–6 GFP's.

Mean values of the dosage groups are compared to the controls. Statistical significance is evaluated by means of the Student' *t*-test.

The percent inhibition of platelet aggregation in the dos-age groups is calculated relative to the vehicle controls.

IC_{50} values (50% inhibition of aggregation) are determined from the concentration-effect curves.

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Markell MS, Fernandez J, Naik UP, Ehrlich Y, Kornecki E (1993) Effects of cyclosporine-A and cyclosporine-G on ADP-stimulated aggregation of human platelets. Ann NY Acad Sci 969: 404–407

B.1.6 Platelet aggregation in whole blood

PURPOSE AND RATIONALE

The method uses a whole blood platelet counter which counts single platelets and does not require their separation from other blood cell types. Platelet aggregation is induced in anti-coagulated human whole blood samples by the addition of the aggregating agents arachidonic acid or collagen. The number of platelets is determined in drug-treated and vehicle control samples; the percentage of inhibition of aggregation and IC_{50} values are calculated in dosage groups. The effect of compounds on other blood cells which secondarily can influence platelet aggregation is included in this test system. The method has been described by Lumley et al. (1981).

PROCEDURE

Materials and solutions

Anticoagulant: sodium citrate to induce platelet aggregation	3.8	%
Sodium arachidonate (Biodata)	3.6×10^{-4}	M
Collagen (Hormonchemie)	10	µg/ml
Serono Hematology System 9000 or Sysmex Micrcellcounter F 800		

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Blood is drawn from healthy adult volunteers, who had not received medication for the last 2 weeks. Nine ml venous blood are anti-coagulated with 1 ml of sodium citrate and kept in a closed tube at room temperature for 30–60 min until the start of the test.

For the aggregation studies, 10 µl test substance or vehicle (controls) are added to 480 µl citrated blood. Samples in closed tubes are pre-incubated for 5 min in a 37°C water shaker bath at 75 strokes/min. Ten µl aggregating agent are added and samples are incubated for another 10 min. The number of platelets (platelet count) is determined in 10 µl samples immediately before and 10 min after the addition of the aggregating agent ('initial platelet count', '10-min-platelet count') in a hematology cell counter.

The following samples for the determination of the platelet count are prepared in duplicate:

- control aggregation = spontaneous aggregation (without aggregating agent):
  480 µl blood + 20 µl vehicle
  Blood samples with >20% spontaneous aggregation are not used to test for induced aggregation.
- maximal aggregation:
  480 µl blood + 10 µl vehicle + 10 µl aggregating agent
  Values represent the maximal induced aggregation rate of the blood sample.
- test substance aggregation:
  480 µl blood + 10 µl test substance
  + 10 µl aggregating agent

EVALUATION

1. From the samples for maximal aggregation (vehicle), the percentage of maximal aggregation is calculated according to the following formula:

$$\%\ \text{maximal aggregation} = 100 - \frac{\text{10-min-platelet count} \times 100}{\text{initial platelet count}}$$

This value for maximal aggregation is taken as 100%.

2. From the samples for test substance induced aggregation, the percentage of aggregation in dosage groups is calculated according to the following formula:

$$\%\ \text{aggregation} = \frac{\text{10-min-platelet count} \times 100}{\text{initial platelet count}}$$

3. IC_{50} values (50% inhibition of aggregation) are determined from the dose-response curves (log concentration test substance versus % inhibition of aggregation).

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B.1.7 Euglobulin lysis time

PURPOSE AND RATIONALE

The euglobulin lysis time is used as an indicator for the influence of compounds on the fibrinolytic activity in rat blood. The euglobulin fraction of plasma is separated from inhibitors of fibrinolysis by acid precipitation and centrifugation. Euglobulin predominantly consists of plasmin, plasminogen, plasminogen activator and fibrinogen. By addition of thrombin to this fraction, fibrin clots are formed. The lysis time of these clots is determined as a measurement of the activity of activators of fibrinolysis (e.g. plasminogen activators). Thus, compounds can be detected which stimulate the release of tissue-type plasminogen activator from the vessel wall.

PROCEDURE

Rats are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium and placed on a heating pad (37 °C). At the same time, the test solution or the vehicle (controls) is administered intravenously or intra-peritoneally. Twenty-five min later, the animals receive another intraperitoneal injection of 12 mg/kg sodium pentobarbital to keep them in deep narcosis for 45 min.

Plasma preparation

After the test compound is absorbed, blood is withdrawn from the inferior caval vein exposed by a midline excision. Blood (1.8 ml) is removed with a plastic syringe containing 0.2 ml 3.8% sodium citrate solution. The sample is thoroughly mixed, transferred to a plastic tube and immediately immersed in ice. Plasma is prepared by centrifugation at 2 000 *g* for 10 min at 2 °C.

Euglobulin preparation

A 0.5 ml portion of plasma is added to 9.5 ml of ice-cold distilled water; the pH is brought to 5.3 by the addition of 0.13 ml of 1% acetic acid. The diluted plasma is kept on ice for 10 min and the precipitated euglobulin fraction is collected by centrifugation at 2 000 *g* for 10 min at 2 °C. The supernatant is discharged and the remaining fluid is removed by drying the tube on a filter paper for 1 min. The euglobulin precipitate is dissolved in 1 ml of 0.12 M sodium acetate solution.

Euglobulin lysis assay

Aliquots (0.45 ml) of the euglobulin solution are transferred to test tubes, and 0.05 ml thrombin (Test Thrombin, Behring Werke) (25 U/ml) are added. The tubes are transferred to a water bath at 37 °C. The time interval between the addition of thrombin and the complete lysis of the clots is measured.

EVALUATION

The lysis time [min] is determined. ELT is shortened when activators of fibrinolysis are increased.

Percent lysis time is calculated in dosage groups as compared to controls.

Statistical evaluation is performed by means of the Student's *t*-test.

REFERENCES

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B.1.8 Fibrinogen receptor binding[2]

PURPOSE AND RATIONALE

The assay is used to evaluate the binding characteristics of drugs at the fibrinogen receptor. A constant concentration of the radioligand ^{125}I-fibrinogen (30–50 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of gel-filtered human platelets. If the test drug exhibits any affinity to fibrinogen receptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent the test drug is.

Platelets are activated with 10 mmol/l ADP to stimulate the ^{125}I-fibrinogen binding at the glycoprotein IIb-IIIa receptor.

[2] Contribution in the first edition by B. Jablonka.

PROCEDURE

Materials and solutions

Solutions for platelet preparation

Stock solution I	Citrate	0.8	%
	sodium citrate	2.2	%
Stock solution II	NaCl	120	mM
	KCl	2.8	mM
	NaH_2PO_4	10.0	mM
	HEPES	10.0	mM
ACD-buffer	Stock solution I		
	+ glucose	2.45	%
	+ hirudin	0.06	U/ml
Tyrode buffer A	Stock solution II		
	+ $NaHCO_3$	12	mM
Tyrode buffer B	Stock solution II		
	+ $NaHCO_3$	12	mM
	+ glucose	5.5	mM
	+ bovine albumin	0.35	%

Tyrode buffers A and B are degassed by aspiration for approx. 1 h after setting the pH to 7.2.

Tyrode buffer C	Tyrode buffer B (degassed)		
	+ apyrase	40	µg/ml
	+ hirudin	0.06	U/ml
Chromatography column	Acryl glass column (200 × 170 mm, 30 mm diameter), closed with 3 perlon filters, pore sizes 63, 90 and 230 µm, and gauze 50 µm filled with degassed Sepharose CL2B-suspen-sion (Pharmacia LKB); equilibrated with 500 ml degassed Tyrode buffer A (2 ml/min)		

Incubation buffer

Stock solution	NaCl	120	mM
	KCl	2.6	mM
	NaH_2PO_4	0.39	mM
	HEPES	10.0	mM
	$CaCl_2$	0.5	mM
Incubation buffer, pH 7.2	stock solution		
	+ $NaHCO_3$	12	mM
	+ glucose	5.5	mM
	+ human albumin	0.35	%

Glucose solution (in incubation buffer)

Radioligand	^{125}I-fibrinogen specific activity 3.7 Mbq/mg fibrinogen (100 µCi/mg fibrinogen) (Amersham), 1 mg radio-labeled fibrinogen is dissolved in 10 ml incubation buffer	
Non-labeled fibrinogen (mw 340 000, grade L, Sigma; in bidistilled water)	10^{-3}–10^{-10}	M
ADP (in incubation buffer)	10	µM

Gamma-counter (1282 Compugamma CS, LKB)

Preparation of gel-filtered platelets

From a healthy volunteer 200 ml blood is collected. An aliquot of 8.4 ml blood is mixed with 1.4 ml ACD-buffer in polystyrol tubes and centrifuged at 1 000 rpm for 15 min. The resulting platelet rich plasma (PRP) is collected and an aliquot is taken for platelet counting. Ten ml PRP are mixed with 1 ml ACD-buffer (ACD-PRP, pH ~ 6.5); 5 ml portions of ACD-PRP are transferred to plastic tubes and centrifuged at 1 600 rpm for 20 min. The resulting supernatant is decanted, and each pellet is resuspended in 500 µl Tyrode buffer C. An aliquot is taken for platelet counting to calculate the loss of platelets. The platelet suspension is then transferred to the Sepharose-packed column which has been eluated with approx. 100 ml degassed Tyrode buffer B (2 ml/min). The column is closed and eluated with degassed Tyrode buffer B (2 ml/min). The first platelets appear after 18–20 min and are then collected for 10 min in a closed plastic cup. Gel-filtered platelets (GFP) are set to 4×10^8 platelets/ml with Tyrode buffer B and kept at room temperature until the start of the test.

Experimental course

For each concentration, samples are tested in triplicate (test tubes No. 72708, Sarstedt).The total volume of each incubation sample is 500 µl. The concentration of ^{125}I-fibrinogen is constant for all samples (10 µg/500 µl).

Competition experiments

The competition reaction is characterized by one buffer value (bidistilled water) and various concentrations of non-labeled fibrinogen or test compound.

- 100 µl ^{125}I-fibrinogen
- 100 µl non-labeled fibrinogen or test drug (various concentrations, 10^{-10}–10^{-3} M)
- 50 µl ADP

Non-specific-binding: The non-specific binding of ^{125}I-fibrinogen is defined as the radioligand binding in the presence of 10^{-5} M of non-labeled fibrinogen.

The binding reaction is started by adding 250 µl GFP (4×10^8 platelets/ml). The samples are incubated for 30 min at room temperature. Subsequently, a 100 µl aliquot of the incubation sample is transferred to a microtainer tube containing 400 µl glucose solution. The tubes are centrifuged at 11 750 rpm for 2 min to separate ^{125}I-fibrinogen bound at the platelet glycoprotein IIb–IIIa receptor from free radio-ligand. The supernatant is carefully decanted and is allowed to run off for approx. 30 min. Radioactivity of the platelet pellets is counted for 1 min in a gamma counter with an efficiency of 65.3%.

EVALUATION

The quantity of the specific ^{125}I-fibrinogen binding results from the difference between the total and the non-specific binding.

Platelet glycoprotein IIb-IIIa receptor binding is given as fmol ^{125}I-fibrinogen/10^8 platelets or ^{125}I-fibrinogen molecules bound per platelet.

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{125}I-fibrinogen versus non-labeled drug by a computer-supported analysis of the binding data.

$$K = \frac{K_D{}^{125}\mathrm{I} \times IC_{50}}{K_D{}^{125}\mathrm{I} + [^{125}\mathrm{I}]}$$

IC_{50} = concentration of the test drug, which displaces 50% of the specifically glyco-protein IIb-IIIa receptor bound ^{125}I-fibrinogen in the competition experiment.

$[^{125}\mathrm{I}]$ = concentration of ^{125}I-fibrinogen in the competition experiment.

$K_D{}^{125}\mathrm{I}$ = dissociation constant of ^{125}I-fibrinogen, determined from the saturation experiment.

The K_i-value of the test drug is the concentration, at which 50% of the fibrinogen receptors are occupied by the test drug.

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B.1.9 PAF antagonism

PURPOSE AND RATIONALE

PAF-antagonism is studied *in vitro* and *in vivo* in the following models (see respective method described):

- platelet aggregation in platelet rich plasma or washed platelets (Sect. B.1.4)

- experimental thrombocytopenia and leucocytopenia (Sect. B.2.9)
- coronary thrombosis in dogs and pigs (Sect. B.2.1)
- bronchospasmolytic activity in anesthetized guinea pigs (Sect. D.2.2.1, D.2.2.2)

Compounds are characterized as PAF antagonists by selective inhibition of PAF-induced effects as compared to effects of other agonists in these models.

B.1.10 Flow behavior of erythrocytes

PURPOSE AND RATIONALE

The deformation of erythrocytes is an important rheological phenomenon in blood circulation. It allows the passage of normal red cells through capillaries with diameters smaller than that of the discoid cells and reduces the bulk viscosity of blood flowing in large vessels. In the following test, the initial flow of filtration is taken as a criterion for erythrocyte deformability. A prolonged time of filtration can be due to 2 basic pathologic phenomena: an increased rigidity of the individual red cells or an increased tendency of the cells to aggregate. To simulate decreased red blood cell deformability, the erythrocytes are artificially modified by one (or by the combination) of the following stress factors:

- addition of calcium ions (increase in erythrocyte rigidity)
- addition of lactic acid (decrease in pH value)
- addition of 350–400 mmol NaCl (hyperosmolarity)
- storing the sample for at least 4 h (cellular ageing, depletion of ADP)

The following procedure can be used to evaluate the effect of test compounds on the flow behavior of erythrocytes.

PROCEDURE

Apparatus

Erythrocyte filtrometer MF 4 (Fa. Myrenne, 52159 Roetgen, Germany)

Membrane filter (Nuclepore Corp.) pore diameter: 5–10 µm, pore density: 4×10^5 pores/cm^2.

Ex vivo

Blood is collected from Beagle dogs, weighing 12–20 kg, or from rabbits, weighing 1.2–2.5 kg, or from Wistar rats, weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In vitro

Following addition of the test compound, blood is incubated at 37 °C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with K-EDTA. (1 mg/ml blood) or heparin (5 IU/ml heparin sodium) and centrifuged at 3 000 rpm for 7 min. The supernatant (plasma) and the buffy coat are removed and discarded. The packed erythrocytes are resuspended in autologous plasma containing 0.25% human albumin and the haematocrit value is fixed at 10%. The red blood cells are altered by one or several of the stress factors mentioned above.

A sample of 2 ml of the stressed suspension is applied to the filtrometer and the initial flow rate is determined. The filtration curve is plotted automatically.

EVALUATION

The cumulative volume of the filtered suspension is recorded per time unit (10 min)

The slope of the curve is determined at different time intervals.

The initial flow rate (10% of the cell suspension having passed the filter) is recorded.

Statistics:
Data of each set are first tested for normal distribution using the Kolmogoroff/Smirnow test. The normal distribution hypothesis is eliminated if the data having a significance level of 5% are not normally distributed. In case that both data sets to be compared are normally distributed the F-test is applied. The hypothesis of homogeneity of variance of both test series is eliminated when the significance level for homogeneity of variance is 5%. The *t*-test for paired and non-paired data is performed when homogeneity of variance is present. In any case, a paired difference test (for paired data) or the U-test (for non-paired data) is likewise carried out (paired of difference test = Wilcoxon test; U-test = Wilcoxon-Mann-Withney or Mann-Whitney test, respectively).

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Teitel P (1977) Basic principles of the 'Filterability test' (FT) and analysis of erythrocyte flow behavior. Blood Cells 3:55–70

B.1.11 Filterability of erythrocytes

PURPOSE AND RATIONALE

The Single Erythrocyte Rigidometer (SER) allows the measurement of deformability of individual red blood cells by determining their passage time through a pore under constant shear stress. In this test, the passage times of single erythrocytes through one pore in a syn-

thetic membrane are determined. The pore in the membrane practically represents a capillary with defined diameter and length. The driving pressure is produced by the constant shear stress. The passage of the red blood cells is measured with the help of an electrical device. A constant current of 50–200 nA is applied. When an erythrocyte passes through the pore the current is interrupted. The test is used to detect compounds that improve filterability of erythrocytes. To simulate decreased red blood cell deformability, the erythrocytes are artificially modified either by one or by a combination of the following stress factors:

- addition of calcium ions (increase in erythrocyte rigidity)
- addition of lactic acid (decrease in pH value)
- addition of 350–400 mmol NaCl (hyperosmolarity)
- storing the sample for at least 4 h (cellular ageing, depletion of ADP)

PROCEDURE

Apparatus

Single erythrocyte rigidometer (Myrenne, 52159 Roetgen, Germany)

Data:
driving pressure: $dp = 70$ Pa (dog, rabbit, rat),
$dp = 100$ Pa (man),
wall shear stress: $\tau = 3$ Pa

Single pore membrane:
length: 30 µm
diameter: 3.5 µm (rat)
4.0 µm (rabbit, dog)
4.5 µm (man)

Ex vivo

Blood is collected from Beagle dogs, weighing 12–20 kg, or from rabbits, weighing 1.2–2.5 kg, or from Wistar rats, weighing 150–300 g, or from man. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In vitro

Following addition of the test compound, the blood samples are incubated at 37ºC for 5 or 30 min.

The blood samples are mixed with K-EDTA (1 mg/ml blood) or heparin (5 IE/ml heparin sodium) to prevent clotting. The blood is centrifuged at 3 000 rpm for 7 min. The plasma and the buffy coat are removed and discarded. The packed erythrocytes are resuspended in filtrated HEPES-buffer containing 0.25% human albumin and the haematocrit value is fixed to < 1%. The red blood cells are altered by one or several stress factors mentioned above. A sample of 2 ml of the stressed suspension is applied to the measuring device and the passage time of a population of 250 erythrocytes (tm) is determined. Cells remaining in the pore for more than 100 ms (tm > 100 ms) lead to a rheological occlusion.

Untreated red blood cell suspensions serve as control.

EVALUATION

The mean passage time of 250 single erythrocytes and the number of rheological occlusions/250 erythrocytes is determined.

Statistics:
The statistical significance is evaluated according to the procedure described for flow behavior of erythrocytes described above.

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B.1.12 Erythrocyte aggregation

PURPOSE AND RATIONALE

The aggregation of red blood cells into rouleaux and from rouleaux into 3-dimensional cell networks is a rheological parameter that decisively influences the flow behavior of blood especially in disturbed microcirculation. In the following procedure an apparatus (erythrocyte aggregometer) is used to measure erythrocyte aggregation. The transparent measuring chamber (cone/ plate configuration) is transilluminated by light of a defined wave length. The intensity of the transmitted light, which is modified by the aggregation process, is recorded. The method can be used to determine the effect of test compounds on erythrocyte aggregation.

PROCEDURE

Apparatus

Selective Erythrocyte Rigidometer (Fa. Myrenne, 52159 Roetgen, Germany)

Ex vivo

Blood is collected from Beagle dogs, weighing 12–20 kg, or from rabbits, weighing 1.2–2.5 kg, or from Wistar rats, weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In vitro

Following addition of the test compound, the blood sample is incubated at 37 °C for 5 or 30 min.

Blood is obtained from the test subjects by venipuncture and mixed with K-EDTA (1 mg/ml) or heparin (5 IU/ml heparin sodium) to prevent clotting. Erythrocyte aggregation is determined in whole blood of 40% haematocrit. A sample of 40 µl blood is transferred to the measuring device. The red cells are dispersed at a shear rate of 600/s. After 20 s, flow is switched to stasis and the extent of erythrocyte aggregation is determined photometrically.

EVALUATION

Statistics:

The statistical significance is evaluated according to the procedure described for flow behavior of erythrocytes described above.

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B.1.13 Determination of plasma viscosity

PURPOSE AND RATIONALE

One of the principal methods for measuring viscosity is based on the rate of flow of a liquid through an orifice. In this test, a defined volume of plasma is transferred into a capillary viscometer and the efflux time required for the plasma to flow from the upper to the lower mark is measured. Using this procedure, the effect of test compounds on the viscosity of blood plasma can be determined. The test can be carried out either *ex vivo* or *in vitro:*

PROCEDURE

Ex vivo

Beagle dogs weighing 12–20 kg, or rabbits weighing 2.0–3.0 kg or Wistar rats weighing 150–300 of either sex are used as test animals. Likewise, the test procedure can be performed in man. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In vitro

Following addition of the test compound, plasma (obtained as described below) is incubated at 37 °C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with 1 mg/ ml blood K^+-EDTA or heparin sodium (5 IU/ml blood) and centrifuged at 3 000 rpm for 5 min. The supernatant (plasma) is removed and a sample of 0.9 ml plasma is transferred into a capillary viscometer (Coulter Harkness, Coulter Electr. LTD, England) provided with a glass capillary of 0.5 mm inside diameter. The temperature during measurement is 37 °C. The flow time t, required for the plasma to flow through the capillary is measured. Untreated plasma serves as control.

EVALUATION

The viscosity of each sample can be determined using the following formula:

$$\eta = K \times t \times \rho$$

η = viscosity of plasma
K = calibration constant of viscometer
t = flow time of 0.9 ml plasma
ρ = density of plasma

The change in viscosity relative to the control group is determined.

Statistical evaluation is carried out using the Student's t-test.

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B.2 *In vivo* or *ex vivo* models

GENERAL CONSIDERATIONS

The general understanding of the pathophysiology of thrombosis is based on the observations of Virchow in 1856. He proposed three factors responsible for thrombogenesis: obstruction of blood flow, changes in the

properties of blood constituents (hypercoagulability), and vessel wall injury. Experimental models of thrombosis focus on one, two or all three factors of Virchow's triad. Therefore, they differ with respect to the prothrombotic challenge – either stenosis, stasis, vessel wall injury (mechanical, electrical, chemical, photochemical, Laser-light), insertion of foreign surface, or injection of a prothrombotic factor –, with respect to the vessel type, and with respect to the animal species. Roughly, two types of models can be differentiated (Didisheim 1972): (1) models in which thrombi are produced in veins by stasis and/or injection of a procoagulant factor resulting in fibrin rich "red" venous type thrombi. (2) models in which thrombi are produced in arteries by vessel wall injury and/or stenosis resulting in platelet rich "white" mural thrombi. But the differentiation is not strict because platelets and the coagulation system influence each other. Drugs preventing fibrin formation may well act in arterial models and vice versa. Thrombosis models are usually performed in healthy animals. The underlying chronic diseases in men, namely atherosclerosis or thrombophilias are not included in the models. Thus, any model is limited regarding its clinical relevance. The pharmacological effectiveness of a new antithrombotic drug should be studied in more than one animal model. Inspite of these limitations animal models predict clinical effectiveness of drugs for the treatment and prevention of thrombotic diseases fairly well. A list of such drugs is presented in a recent review by Leadleey et al. (2000).

Furthermore, the clinical usefulness of an antithrombotic drug is determined by its safety/efficacy ratio regarding the bleeding risk. Assessment of a parameter of the hemostatic system should therefore be included in the models if possible.

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B.2.1 Stenosis- and mechanical injury-induced coronary thrombosis (Folts-model)

PURPOSE AND RATIONALE

Thrombosis in stenosed human coronary arteries is one of the most common thrombotic diseases leading to unstable angina, acute myocardial infarction or sudden death. Treatment with angioplasty, thrombolysis, or by-pass grafts can expose new thrombogenic surfaces and re-thrombosis may occur. The mechanisms responsible for this process include interactions of platelets with the damaged arterial wall and platelet aggregation.

In order to study new drugs for their antithrombotic potential in coronary arteries, Folts (1974) developed the model of periodic acute platelet thrombosis and cyclic flow reductions (CFR's) in stenosed canine coronary arteries. Uchida described a similar model in 1975. The model includes various aspects of unstable angina pectoris, i.e. critical stenosis, vascular damage, downstream vasospasm induced by vasoconstrictors released or generated by platelets. The cyclic variations in coronary blood flow are a result of acute platelet thrombi which may occlude the vessel but which either embolize spontaneously or can easily be embolized by shaking the constricting plastic cylinder. They are not a result of vasospasm (Folts 1982). Clinically, aspirin can reduce the morbidity and mortality of coronary thrombotic diseases but its effect is limited. Similarly, CFR's in the Folts model are abolished by aspirin but the effect can be reversed by increases in catecholamines and shear forces (Folts 1988). As part of an expert meeting on animal models of thrombosis, a review of the Folts model has been published (Folts 1991).

Five different protocols are described in the following section for the induction of coronary thrombosis.

Coronary thrombosis induced by stenosis

The described preparations are characterized by episodic, spontaneous decreases in coronary blood flow interrupted by restorations of blood flow. These alterations in coronary blood flow, called cyclic flow reductions (CFR), are associated with transient platelet aggregation at the site of the coronary constriction and abrupt increase in blood flow after embolization of platelet-rich thrombi.

Damage of the vessel wall is produced by shortly placing a hemostatic clamp on the coronary artery; a fixed amount of stenosis is produced by an externally applied obstructive plastic cylinder upon the damaged part of the vessel. In dogs, the stenosis is critical, i.e. the reactive hyperemic response to a 10 second-occlusion is abolished (protocol 1); in pigs, the stenosis is subcritical, i.e. there is a partial reactive hyperemia left (Just et al. 1991; protocol 2).

For some animals, especially for young dogs, damage of the vessel wall and stenosis are not sufficient to induce thrombotic cyclic flow variations. In these cases, an additional activation of platelets by infusion of epinephrine (protocol 3) is required leading to the formation of measurable thrombi. In another preparation (protocol 4), thrombus formation is induced by subcritical

stenosis without prior clamping of the artery and infusion of platelet activating factor (PAF) according to the model described by Apprill et al. (1985). In addition to these protocols, coronary spasms induced by released platelet components can influence coronary blood flow. Therefore, this model includes the main pathological factors of unstable angina pectoris.

Coronary thrombosis induced by electrical stimulation

In this preparation, coronary thrombosis is induced by delivery of low amperage electrical current to the intimal surface of the artery according to the method described by Romson et al. (1980a). In contrast to the stenosis protocols, an occluding thrombosis is formed gradually without embolism after some hours (protocol 5). As a consequence of this time course, the thrombi formed are of the mixed type and contain more fibrin than the platelet thrombi with critical stenosis.

PROCEDURE

Coronary thrombosis induced by stenosis

Protocol 1: Critical stenosis

Dogs of either sex weighing 15–40 kg, at least 8 months of age, are anesthetized with pentobarbital sodium (bolus of 30–40 mg/kg and continuous infusion of approx. 0.1 mg/kg/min); respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth or fifth intercostal space, the pericard is opened and the left circumflex coronary artery (LCX) is exposed. An electromagnetic or Doppler flowprobe is placed on the proximal part of the LCX to measure coronary blood flow. Distal to the flowprobe, the vessel is squeezed with a 2 mm hemostatic clamp for 5 s. A small cylindrical plastic constrictor, 2–4 mm in length and with an internal diameter of 1.2 to 1.8 mm (depending on the size of the LCX) is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times (2–5 times) until the appropriate narrowing of the vessel is achieved, and cyclic flow variations are observed. In case of an occlusion of the artery without spontaneous embolization of the formed thrombus, reflow is induced by shortly lifting the vessel with a thread placed beneath the stenotic site.

Only dogs with regularly repeated cyclic flow reductions (CFR's) of similar intensity within a pre-treatment phase of 60 min are used in these experiments.

The test substance is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application. CFR's are registered for 2 to 4×60 min and compared to pre-treatment values.

Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

Protocol 2: Subcritical stenosis

Male castrated pigs (German landrace, weighing (20–40 kg) are anesthetized with ketamine (2 mg/kg i.m.), metomidate (10 mg/kg i.p.) and xylazine (1–2 mg/kg i.m.). In order to maintain the stage of surgical anesthesia, animals receive a continuous i.v. infusion of 0.1–0.2 mg/kg/min pentobarbital sodium. Respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth and fifth intercostal space, the pericard is opened and the left descending coronary artery (LAD) is exposed. An electromagnetic or Doppler flowprobe is placed on the proximal part of the LAD to measure coronary blood flow. Distal to the flowprobe, the vessel is squeezed with a 1 mm hemostatic clamp for 5 s. A small cylindrical plastic constrictor, 2 mm in length, is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times until the appropriate narrowing of the vessel is achieved, which produces cyclic flow reductions. CFR's are similar to those in dogs; pigs, however, show a reactive hyperemic response. If embolization does not occur spontaneously, the formed thrombus is released at 2/3 reduction of blood flow by shortly lifting the vessel with forceps.

Only pigs with regularly repeated CFR's of similar intensity within a pre-treatment phase of 60 min are used in these experiments.

The test substance is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application. CFR's are registered for 2×60 min and compared to pre-treatment values.

Protocol 3: Stenosis + epinephrine infusion

If protocol 1 does not lead to CFR's, additionally epinephrine (0.2 µg/kg/min) is infused into a peripheral vein for 2×60 min (60 min before and 60 min following drug administration). CFR's are registered and compared in the 60 min post-drug phase to the 60 min pre-drug phase.

Protocol 4: Stenosis + PAF infusion

The LCX is stenosed without prior mechanical wall injury. This preparation does not lead to thrombus formation (subcritical stenosis). For the induction of CFR's, in addition PAF (C 16-PAF, Bachem) (0.2 nmol/kg/min) is infused into one cannulated lateral branch of the coronary artery.

After 30 min, PAF infusion is terminated and blood flow returns to its normal, continuous course. Thirty min later, concomitantly the test substance is administered and a second PAF infusion is started for 30 min.

CFR's are registered and compared in the drug treated, second PAF phase to the pre-drug, first PAF phase.

Coronary thrombosis induced by electrical stimulation

Protocol 5
The LCX is punctuated distal to the flow probe with a chrome-vanadium-steel electrode (3 mm length, 1 mm diameter). The electrode (anode) is placed in the vessel in contact with the intimal lining and connected over a Teflon coated wire to a 9 Volt battery, a potentiometer and an amperemeter. A disc electrode (cathode) is secured to a subcutaneous thoracal muscle layer to complete the electrical circuit. The intima is stimulated with 150 µA for 6 h. During this time, gradually an occluding thrombosis is formed.

The test substance or the vehicle (control) is administered either at the start of the electrical stimulation or 30 min following the start.

The time interval until the thrombotic occlusion of the vessel occurs and the thrombus size (wet weight measured immediately after removal at the end of the experiment) are determined.

Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

For all protocols the following preparations and measurements are performed:

- To measure peripheral arterial blood pressure (BP) [mm Hg], the right femoral artery is cannulated and connected to a Statham pressure transducer.
- Left ventricular pressure (LVP) [mm Hg] is determined by inserting a micro-tip-catheter via the carotid artery retrogradely.
- Left ventricular enddiastolic pressure (LVEDP) [mm Hg] is evaluated through sensitive amplification of the LVP.
- Contractility (LV *dp*/*dt* max) [mm Hg/s] is determined from the initial slope of the LVP curve.
- Heart rate [min^{-1}] is determined from the pulsatile blood pressure curve.
- The ECG is recorded in lead II.
- Arterial pH and concentrations of blood gases are kept at physiological levels by adjusting respiration and infusion of sodium bicarbonate.
- Blood hematocrit values (37–40%) and number of erythrocytes are kept constant by infusion of oxypolygelatine in dogs and electrolyte solution in pigs.
- Body temperature is monitored with a rectal thermistor probe and kept constant by placing the animals on a heated metal pad with automatic regulation of temperature.
- Template buccal mucosal bleeding time using the Simplate® device

At the end of the test, animals are sacrificed by an overdose of pentobarbital sodium.

EVALUATION
For all protocols, the mean maximal reduction of blood pressure (systolic/diastolic) [mm Hg] is determined.

Protocol 1–4
The following parameters are determined to quantify stenosis-induced coronary thrombosis:

- Frequency of cyclic flow reductions
  = cycle number per time
- Magnitude of cyclic flow reductions
  = cycle area [mm^2] (total area of all CVR's per time, measured by planimetry)

Percent change in cycle number and cycle area after drug treatment is calculated compared to pre-treatment controls.

Statistical significance is assessed by the paired Student's *t*-test.

Protocol 5
The following parameters are determined to quantify electrically-induced coronary thrombosis:

- Occlusion time [min]
  = time to zero blood flow
- Thrombus size [mg]
  = wet weight of the thrombus immediately after removal

Percent change in mean values for occlusion time and thrombus size in drug-treated groups is compared to the control group.

Statistical significance is assessed by the non-paired Student's *t*-test.

Standard data:

Standard	Dose	% decrease in CFV's		Protocol	Species
		Number	Area		
Acetylsalicylic acid	1 mg/kg, i.v.	56	80	1	dog
	20 mg/kg, i.d.	87	95	1	dog
	30 mg/kg, i.v.	56	77	2	pig

CRITICAL ASSESSMENT

Both, the stenosis (Folts) and the electrical (Romson/Lucchesi) model of coronary thrombosis are widely used to study the role of mediators in the thrombotic process and the effect of new antithrombotic drugs. Bush (1986) reviewed the role of the endothelium in arterial thrombosis and the effect of some inhibitors and mediators in the Folts model, e.g. thromboxane, prostacyclin, cyclooxigenase, serotonin, NO-donors and other vasodilators. The effect of an NO-donor could be reversed by the NO-scavenger oxyhemoglobin indication that indeed NO was responsible for the antithrombotic action (Just et al. 1991). Recent mechanisms of antithrombotic drug action which have been studied in either of the two coronary thrombosis models are: the oral GP IIb/IIIa antagonist DMP 728 (Mousa et al. 1996); the LMWH enoxaparin (Leadley et al. 1998) which inhibited CFR's in contrast to unfractionated heparin; the thrombin-inhibitors PEG-hirudin (Ruebsamen et al. 1998) and melagatran (Mehta et al. 1998); an anti-P-selectin antibody (Ikeda et al. 1999); and activated protein C (Jackson et al. 2000).

The clinical relevance of studies in the Folts-model has been questioned because the model is very sensitive to antithrombotic compounds. However, the lack of a reversal of the effect by epinephrine or increase in degree of stenosis differentiates any new drug from aspirin. Electrical coronary thrombosis is less sensitive: e.g. aspirin has no effect and with some drugs higher dose levels are required; but in principle, most drug mechanisms act in both models if at all.

MODIFICATIONS OF THE METHOD

Romson et al. (1980b) described a simple technique for the induction of coronary artery thrombosis in the conscious dog by delivery of low amperage electric current to the intimal surface of the artery.

Benedict et al. (1986) modified the electrical induction of thrombosis by use of two Doppler flow probes proximal and distal to the needle electrode in order to measure changes in blood flow velocity. The electrical current was stopped at 50% increase in flow velocity and thrombosis then occurred spontaneously. The important role of serotonin was demonstrated by increases in coronary sinus serotonin levels just prior to occlusion.

Warltier et al. (1987) described a canine model of thrombin-induced coronary artery thrombosis: and the effects of intracoronary streptokinase on regional myocardial blood flow, contractile function, and infarct size.

Al-Wathiqui et al. (1988) described the induction of cyclic flow reduction in the coronary, carotid, and femoral arteries of conscious chronically instrumented dogs.

The method of Folts-thrombosis has also been applied to carotid arteries in **monkeys**. Coller et al. (1989) induced CFR's in carotid arteries of anesthetized cynomologus monkeys and showed abolition by the GP Iib/IIIa antibody abciximab.

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B.2.2
Stenosis-and mechanical injury-induced arterial and venous thrombosis (Harbauer-model)

PURPOSE AND RATIONALE

Harbauer (1984) first described a venous model of thrombosis induced by mechanical injury and stenosis of the jugular vein. In a modification, both arterial and venous thrombosis is produced in rabbits by stenosis of the carotid artery and the jugular vein with simultaneous mechanical damage of the endothelium. This activates platelets and the coagulation system and leads to changes in the bloodstream pattern. As a consequence, occluding thrombi are formed as detected by blood flow measurement. The dominant role of platelets in this model is shown by the inhibitory effect of an antiplatelet serum in both types of vessels (Just 1986). The test is used to evaluate the antithrombotic capacity of compounds in an *in vivo*-model of arterial and venous thrombosis where thrombus formation is highly dependent on platelet activation.

PROCEDURE

Male Chinchilla rabbits weighing 3–4 kg receive the test compound or the vehicle (controls) by oral, intravenous or intraperitoneal administration. The first ligature (vein, preparation see below) is performed at the end of absorption (i.p. approx. 30 min, p.o. approx. 60 min, i.v. variable).

Sixty-five min before stenosis, the animals are sedated by intramuscular injection of 8 mg/kg xylazine (Rompun®) and anesthetized by intravenous injection of 30–40 mg/kg pentobarbital sodium 5 min later. During the course of the test, anesthesia is maintained by continuous infusion of pentobarbital sodium (30–40 mg/kg/h) into one femoral vein.

A Statham pressure transducer is placed into the right femoral artery for continuous measurement of blood pressure. Spontaneous respiration is maintained through a tracheal tube.

One jugular vein and one carotid artery are exposed on opposite sides. Small branches of the vein are clamped to avoid blood flow in spite of vessel occlusion.

Electromagnetic or Doppler flow probes are placed on the vein (directly central to the vein branching) and on the artery (as far central as possible). Blood flow [ml/min] is measured continuously.

After reaching steady state (approx. 15–30 min), a metal rod with a diameter of 1.3 mm is placed on the jugular vein (2 cm central to the vein branching) and a ligature is tightened. After 1 min, the rod is removed from the ligature. Immediately thereafter (approx. 1.5 min), the carotid artery is damaged by shortly squeezing it with forceps. Then a small plastic constricting cylinder with 1.2 mm diameter and 2 mm length is placed around the site of the endothelial damage.

Registration of parameters is terminated after 30 min.

In addition, the template bleeding time is measured at various time interval before and after drug treatment (depending on the route of administration) in the shaved inner ear using the Simplate® device. Care is taken to select parts of the skin without larger vessels.

EVALUATION

Percent thrombus formation (= thrombosis incidence) is judged by determination of the number of occluded vessels (blood flow = 0).

Percent inhibition of thrombosis incidence is calculated in dosed groups as compared to vehicle controls.

Thrombosis incidence is always 100% in vehicle controls.

Statistical significance is assessed by means of the Fisher-exact-test.

If initial values for blood flow do not significantly differ in dosage and control groups, the area below the blood flow curves is measured by planimetry in addition and mean values in dosed groups are compared to controls by means of the unpaired Student's *t*-test.

Mean values of occlusion times [min] in dosage and control groups are calculated and compared by means of the t-test.

The maximal change in systolic and diastolic blood pressure during the time period of stenosis as compared to the initial values before drug administration is determined. There is no standardized assessment score. As an example, a reduction of systolic blood pressure by 30 mm Hg and of diastolic blood pressure by 20 mm Hg is quoted as a strong reduction in blood pressure.

CRITICAL ASSESSMENT

Two main factors of arterial thrombosis in men are essential in this model: high grade stenosis and vessel wall damage. In the absence of either no thrombus is found. The occlusive thrombus is formed fast and in a highly reproducible manner. In both vessels thrombus formation is equally dependent on platelet function as shown by antiplatelet serum. Therefore, the jugular vein thrombosis in this model differs from stasis-induced deep vein thrombosis with predominant fibrin formation. On the other hand, these occlusive thrombi are more stable than the pure platelet thrombi in the Folts model (see B.2.1) since carotid blood flow cannot be restored by shaking the constrictor. The following antithrombotic drugs are effective: i) antiplatelet drugs like ticlopidine, prostacyclin/iloprost, NO-donors (SNP, molsidomine) but not aspirin, thromboxane-synthase-inhibitors; ii) anticoagulants like hirudin, high-dose heparin, warfarin; iii) streptokinase/t-PA (Bevilacqua 1991; Just 1986 and unpublished). In contrast, drugs which only lower blood pressure such as hydralazine, clonidine, prazosin have no effect on thrombus formation in this model.

MODIFICATIONS OF THE METHOD

Bevilacqua et al. (1991) performed the same model in rabbit carotid arteries but compared the procedure in one artery before drug treatment with the contralateral artery after drug treatment. Heparin, the synthetic thrombin inhibitor FPRCH2Cl, iloprost and t-PA inhibited carotid occlusion in this model but not aspirin.

Spokas and Wun (1992) produced venous thrombosis in the vena cava of rabbits by vascular damage and stasis. The vascular wall was damaged by crushing with hemostat clamps. A segment of the vena cava was looped with two ligatures, 2.5 cm apart. At 2 h after ligation, the isolated venous sac was dissected and the clot removed for determination of dry weight.

Lyle et al. (1995) searched for a animal model mimicking the thrombotic re-occlusion and restenosis occurring in several cases after successful coronary angioplasty in man. The authors developed a model of angioplasty-induced injury in atherosclerotic rabbit femoral arteries. Acute ^{111}indium-labelled platelet deposition and thrombosis were assessed four hours after balloon-injury in arteries subjected to prior endothelial damage (air desiccation) and cholesterol supplementation (one month). The effects of inhibitors of factor X_a or platelet adhesion, heparin, and aspirin on platelet deposition were studied.

Thrombosis induced by supercooling

Meng (1975), Meng and Seuter (1977) and Seuter et al. (1979) described a method to induce arterial thrombosis in rats by chilling of the carotid artery. Rats were anesthetized, the left carotid artery was exposed and occluded proximal by means of a small clamp. The artery was placed for 2 min into a metal groove which was cooled to –15 °C. The vessel was compressed by a weight of 200 g. In addition, a silver clip was fixed to the vessel distally from the injured area to produce a disturbed and slow blood flow. After 4 min, the proximal clamp was removed and the blood flow reestablished in the injured artery. In the rabbit, slightly different conditions were used: the chilling temperature was –12 °C for a period of 5 min, and the compressing weight was 500 g. The wound was closed and the animal allowed to recover from anesthesia. Antithrombotic compounds were administered in various doses at different time intervals before surgery. After 4 h, the animals received heparin and were re-anesthetized. The lesioned carotid artery was removed and thrombus wet-weight was immediately measured.

The method has been modified by Granzer (1986) using a cryo-forceps perfused with methanol at –21 °C by which the exposed carotid artery or jugular vein was compressed over a length of 12 mm with a load of 500 g for two min.

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B.2.3 Electrical-induced thrombosis

PURPOSE AND RATIONALE

The use of electrical current to induce thrombosis in hamster and dog has been described in the early 1950s by Lutz et al. (1951) and Sawyer and Pate (1953a,b). In general, two different approaches exist. One method produces electrical damage by means of two externally applied hook-like electrodes (Hladovec 1973; Philp et al. 1978). The other method uses a needle electrode which is advanced through the walls of the blood vessels and positioned in their lumen, the second electrode is placed into a subcutaneous site completing the circuit (Salazar 1961; Romson et al. 1980; Benedict et al. 1986).

PROCEDURE

Anaesthetized rats weighing 200–300 g are intubated and a femoral artery is cannulated for administration of drugs. One carotid artery is isolated from surrounding tissues over a distance of 10–15 mm.

A pair of rigid stainless-steel wire hook-like electrodes with a distance of 4 mm are adjusted to the artery by means of a rack and pinion gear manipulator. The artery is raised slightly away from the surrounding tissue. Isolation of the electrodes is achieved by the insertion of a small piece of parafilm under the artery. Blood flow is measured with an ultrasonic Doppler flowmeter (Transonic, Ithaca NY, USA), the flow probe (1RB) is placed proximal to the damaged area.

Thrombus formation is induced in the carotid arteries by the application of an electrical current (350 V, DC, 2 mA) delivered by an electrical stimulator (Stoelting Co, Chicago, Cat. No 58040) for 5 min to the exterior surface of the artery.

EVALUATION

- Blood flow before and after induction of thrombus for 60 min
- Time to occlusion [min]: the time between onset of the electrical current and the time at which blood flow decreases under 0.3 ml/min
- Patency of the blood vessel over 30 min

CRITICAL ASSESSMENT

The electrical-induced thrombus is composed of densely packed platelets with some red cells. Moreover, the electrical injury causes extensive damage to intimal and subintimal layers. The endothelium is completely destroyed and this damage extends to subendothelial structures including smooth muscle cells. The deep damage could reduce the possibility of discrimination between drugs on the basis of their antithrombotic activity. However, Philp et al. (1978) could show that unfractionated heparin completely blocked thrombus formation, whereas other antiplatelet agents displayed differentiated antithrombotic action. He concluded that this relatively simple model of arterial thrombosis might prove a useful screening test for drugs with antithrombotic potential.

MODIFICATIONS OF THE METHOD

The technique described by Salazar et al. (1961) uses a stainless steel electrode which is inserted into a coronary artery in the dog and which delivers anodal current to the intravascular lumen. The electrode is positioned under fluoroscopic control which complicates the method. The technique was modified by Romson et al. (1980). They placed the electrode directly into the coronary artery of open-chest anaesthetized dogs.

Rote et al. (1993, 1994) used a carotid thrombosis model in dogs. A calibrated electromagnetic flow meter was placed on each common carotid artery proximal to both the point of insertion of an intravascular electrode and a mechanical constrictor. The external constrictor was adjusted with a screw until the pulsatile flow pattern decreased by 25% without altering the mean blood flow. Electrolytic injury to the intimal surface was accomplished with the use of an intravascular electrode composed of a Teflon-insulated silver-coated copper wire connected to the positive pole of a 9-V nickel-cadmium battery in series with a 250 000 ohm variable resistor. The cathode was connected to a sub-

cutaneous site. Injury was initiated in the right carotid artery by application of a 150 µA continuous pulse anodal direct current to the intimal surface of the vessel for a maximum duration of 3 h or for 30 min beyond the time of complete vessel occlusion as determined by the blood flow recording. Upon completion of the study on the right carotid, the procedure for induction of vessel wall injury was repeated on the left carotid artery after administration of the test drug.

Benedict et al. (1986) introduced a procedure in which anodal current is discontinued when mean distal coronary flow velocity increased by approximately 50%, reflecting disruption of normal flow by the growing thrombus. Occlusive thrombosis occurred within 1 h after stopping the electrical current. It was observed that the final phase of thrombosis occurred independently of electrical injury.

A ferret model of acute arterial thrombosis was developed by Schumacher et al. (1996). A 10-min anodal electrical stimulation of 1 mA was delivered to the external surface of the carotid artery while measuring carotid blood flow. This produced an occlusive thrombus in all vehicle treated ferrets within 41 ±3 min with an average weight of 8 ±1 mg. Thrombus weight was reduced by aspirin or a thromboxan receptor antagonist.

Guarini (1996) produced a completely occlusive thrombus in the common carotid artery of rats by applying an electrical current to the arterial wall (2 mA for 5 min) while simultaneously constricting the artery with a hemostatic clamp placed immediately downstream from the electrodes.

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B.2.4 $FeCl_3$-induced thrombosis

PURPOSE AND RATIONALE

A variety of chemical agents has been used to induce thrombosis in animals. Topical $FeCl_3$ was described by Reimann-Hunziger (1944) as thrombogenic stimulus in veins. Kurz et al. (1990) showed that the thrombus produced with this method in the carotid arteries of rats is composed of platelets and red blood cells enmeshed in a fibrin network. This model is used as a simple and reproducible test for evaluation of antithrombotic (Broersma et al. 1991) and profibrinolytic test compounds (van Giezen et al. 1997).

PROCEDURE

Rats weighing between 250 and 300 g are anaesthetized with Inactin (100 mg/kg) and a polyethylene catheter (PE-205) is inserted into the trachea via a tracheotomy to facilitate breathing. Catheters are also placed in the femoral artery for blood samples and measurement of arterial blood pressure and in the jugular vein for administration of test agents. The right carotid artery is isolated and an ultrasonic Doppler flowprobe (probe 1RB, Transonic, Ithaca NY, USA) is placed on the vessel to measure blood flow. A small piece of Parafilm "M" (American Can Co., Greenwich, CT) is placed under the vessel to isolate it from surrounding tissues throughout the experiment.

The test agent is administered by gavage or as an intravenous injection at a defined time prior to initiation of thrombus formation. Thrombus formation is induced by the application of filter paper (2×5 mm), saturated with 25% $FeCl_3$ solution, to the carotid artery. The paper is allowed to remain on the vessel 10 min before removal. The experiment is continued for 60 min after the induction of thrombosis. At that time, the thrombus is removed and weighed.

EVALUATION

- Blood flow before and after induction of thrombus for 60 min

- Time to occlusion [min]: the time between $FeCl_3$ application and the time at which blood flow decreases under 0.3 ml/min
- Thrombus weight after blotting the thrombus on filter paper

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B.2.5 Laser-induced thrombosis

PURPOSE AND RATIONALE

Thrombus formation in rat or rabbit mesenteric arterioles or venules is induced by laser beams. The test can be performed in normal or pretreated (induction of arteriosclerosis or adjuvant arthritis) animals. The mediators for thrombus formation in this method are platelet adhesion to the injured endothelial vessel wall on one hand and ADP-induced platelet aggregation on the other. Most probably, ADP is primarily released by laser beam lysed erythrocytes, due to the fact that erythrocyte hemoglobin exerts strong adsorbability to frequencies emitted by laser beams. There is a further, secondary, aggregation stimulus following the release reaction induced by the platelets themselves.

PROCEDURE

Apparatus

4W Argon laser (Spectra Physics, Darmstadt, FRG) wave length: 514,5 nm, energy below the objective: 15 mW, duration of exposure: 1/30 or 1/15 s

- Microscope ICM 405, LD-Epipland 40/0.60 (Zeiss, Oberkochen, FRG)
- Video camera (Sony, Trinicon tube)
- Recorder (Sony, U-matic 3/4")
- Videoanalyzer and correlator to determine blood flow velocity

In vivo experiment

Male Sprague Dawley or spontaneously hypertensive stroke prone Wistar or Lewis rats with adjuvant induced arthritis weighing 150–300 g or New Zealand rabbits with arteriosclerosis induced by cholesterol feeding for 3 months are used. The animals receive the test compound by oral, intravenous, intraperitoneal, or subcutaneous administration. Control animals are treated with vehicle alone. Prior to thrombus induction, the animals are pretreated by s.c. injection of 0.1 mg/kg atropine sulfate solution and anaesthetized by intraperitoneal administration of 100 mg/kg ketamine hydrochloride and 4 mg/kg xylazine.

Thrombus formation is induced 15, 30, 60 or 90 min post dosing. Investigations are performed in arterioles or venules of 13 ±1 µm in diameter of the fat-free ileocaecal portion of the mesentery. During the test procedure, the mesenterium is superfused with physiological saline solution or degassed paraffin liquid (37 °C). The ray of the argon laser is led into the inverted ray path of the microscope by means of a ray adaptation and adjusting device. The frequency of injuries is 1 per 2 min. The exposure time for a single laser shot is 1/30 or 1/15 s. The number of injuries necessary to induce a defined thrombus is determined. All thrombi formed during the observation period with a minimum length of 13 µm or an area of at least 25 μm^2 are evaluated. All measuring procedures are photographed by a video system.

Standard compounds:

- acetylsalicylic acid (10 mg/kg, per os)
- pentoxifylline (10 mg/kg, per os)

EVALUATION

The number of laser shots required to produce a defined thrombus is determined. Mean values and SEM are calculated. Results are shown graphically.

For statistical evaluation the χ^2-test is used.

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B.2.6 Photochemical-induced thrombosis

PURPOSE AND RATIONALE

In 1977, Rosenblum and Sabban reported that ultraviolet light can produce platelet aggregation in cerebral microvessels of the mouse after intravascular administration of sodium fluorescein. They found that in contrast to heparin, both aspirin and indomethacin prolonged the time to first platelet aggregate. Herrmann (1983) provided a detailed study in which he showed that scavengers of singlet oxygen, not of hydroxyl radicals, inhibited platelet aggregation induced by the photochemical reaction. He postulated that by exciting the intravascularly administrated fluorescein, singlet oxygen damages endothelial cells which subsequently leads to platelet adhesion and aggregation.

PROCEDURE

Studies are performed in mesenteric arteries of 15–30 μm diameter in anesthetized rats. After intravenous injection of fluorescein isothiocyanate-dextran 70 (FITC-dextran, Sigma, 10%, 0.3 ml), the FITC-dextran in arterioles is exposed to ultraviolet light (wavelength of excitation 490 nm, wavelength of emission 510 nm).

EVALUATION

Thrombus formation is quantitated by determining the time between onset of excitation and appearance of the first platelet aggregate adhering to the vessel wall.

MODIFICATIONS OF THE METHOD

Matsuno et al. (1991) reports a method to induce thrombosis in the rat femoral artery by means of a photochemical reaction after injection of a fluorescent dye (rose Bengal, 10 mg/kg i.v.) and transillumination with a filtered xenon lamp (wave length: 540 nm). Blood flow is monitored by a pulsed Doppler flow meter. Occlusion is achieved after approximately 5–6 min. Pretreatment with heparin dose-dependently prolongs the time required to interrupt the blood flow. The model also enables to study thrombolytic mechanisms, which had been evaluated with t-PA.

CRITICAL ASSESSMENT

In contrast to other thrombosis induction methods, photochemically-induced thrombosis can be easily used in smaller animals. Thrombi are composed primarily of platelets, however the primary target of the photochemical insult is the endothelial cells by means of a oxygen radical damage. So, it might be disadvantageous that beneath platelet inhibitors also scavenger are effective.

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B.2.7 Foreign-surface thrombosis

The presence of foreign materials in the circulation produces activation of the coagulation and the platelet system. Various prothrombotic surfaces have been used to develop experimental animal models. In contrast to many other thrombosis models, the thrombosis induced by foreign surfaces does not presuppose endothelial damage.

B.2.7.1 Wire-coil induced thrombosis

PURPOSE AND RATIONALE

A classical method to produce thrombosis is based on the insertion of wire coils into the lumen of blood vessels. The model was first described by Stone and Lord (1951) in aorta of dog's and was further modified to be used in arterial coronary vessels of opened-chest dogs. The use in venous vessels was described by Kumada et al. (1980).

The formation of thrombotic material around the coil is reproducible and can be easily standardized to study pharmacological agents (Just and Schönafinger 1991; Mellott et al. 1993; Rübsamen and Hornberger 1996).

Venous thrombosis is produced in rats by insertion of a stainless steel wire coil into the inferior caval vein. Platelets as well as plasmatic coagulation are activated on the wire coil. Thrombus formation onto the wire is quantitated by measuring the protein content of the thrombotic material isolated. The kinetics of thrombus formation show an increase in weight and protein content within the first 30 min followed by a steady state between thrombus formation and endogenous thrombolysis leading to a constant protein content of thrombi between 1 and up to 48 h following implantation of the wire coil. Thrombosis incidence in untreated control ani-

mals in this model is 100%. The test is used to evaluate antithrombotic and thrombolytic properties of compounds in an *in vivo*-model of venous thrombosis in rats.

PROCEDURE

Male Sprague-Dawley rats weighing 260–300 g receive the test compound or the vehicle (controls) by oral, intravenous or intraperitoneal administration. At the end of absorption (i.v. 1 min, i.p. 30 min, p.o. 60 min), the animals are anesthetized by intraperitoneal injection of 1.3 g/kg urethane. Through a mid-line incision the caudal caval vein is exposed and a stainless steel wire coil (a dental pate carrier, Zipperer® size 40(st), Zdarsky Erler KG, München) is inserted into the lumen of the vein just below the left renal vein branching by gently twisting of the wire towards the iliac vein. The handle of the carrier is cut off so as to hold the back end of the wire at the vein wall. The incision is sutured and the animal is placed on its back on a heating pad (37 °C). The wound is reopened after 2 h, the wire coil is carefully removed together with the thrombus on it and rinsed with 0.9% saline. The thrombotic material is dissolved in 2 ml alkaline sodium carbonate solution (2% Na_2CO_3 in 0.1 N NaOH) in a boiling water bath for 3 min. The protein content is determined in 100 µl aliquots by the colorimetric method of Lowry.

Thrombolysis

In addition to the described preparation, for continuous infusion of a thrombolytic test solution a poly-ethylene catheter is inserted in the jugular vein. One and a half h after implantation of the wire coil, the test compound or the vehicle (controls) is infused for up to 2.5 h. The wire coil is then removed and the protein content of thrombi is determined (see above). Bernat et al. (1986) demonstrated the fibrinolytic activity of urokinase and streptokinase-human plasminogen complex in this model.

EVALUATION

Thrombosis incidence (= number of animals with thrombi in dosage groups as compared to vehicle controls) is assessed.

The mean protein content [mg] of the thrombotic material in dosage groups and vehicle controls is determined. Percent change in protein content is calculated in dosage groups as compared to controls.

Statistical significance is assessed by means of the unpaired Student's *t*-test.

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B.2.7.2 Eversion-graft induced thrombosis

PURPOSE AND RATIONALE

The eversion graft model for producing thrombosis in the rabbit artery was first described by Hergrueter et al. (1988) and later modified by Jang et al. (1989, 1990) and Gold et al. (1991). A 4- to 6-mm segment of the rabbit femoral or the dog left circumflex artery is excised, everted and then re-implanted into the vessel by end-to-end anastomoses. After restoration of the blood flow, a platelet-rich occlusive thrombus forms rapidly leading to complete occlusion of the vessel. This model mimics a deep arterial injury since the adventitial surface is a non-endothelial tissue containing tissue factor and collagen. The rabbit model described here uses a carotid graft inserted into the femoral graft to avoid vasoconstriction often occurring in the inverted femoral segments.

PROCEDURE

In anaesthetized New Zealand White rabbits, the right A. carotis is exposed. After double ligation, a 3 mm segment of the artery is excised, everted and immersed in pre-warmed (37 °C) isotonic saline. Thereafter, the right femoral artery is exposed and occluded by means of a double-occluder (2 cm distance). The femoral artery is transected and the everted graft from the carotid artery is inserted by end-to-end anastomosis using 12 sutures with 9-0 nylon (Prolene, Ethicon, Norderstedt, Germany) under a surgical microscope (Wild M650, Leitz, Heerbrugg, Switzerland). Perfusion of the graft is measured by means of an ultrasonic flowmeter (Model T106, Transonic, Ithaca NY, USA), The flow probe is positioned 2 cm distal from the graft.

After a stabilization period of 15 min, the test substance is given intravenously through the catheterized right V. jugularis. 10 min after substance administration, the vessel clamps are released and the blood flow is monitored by the flowmeter for 120 min.

Arterial blood is collected from the left carotid artery at baseline (immediately before substance administration), 10 min, 60 min and 120 min after substance administration.

EVALUATION

- Time until occlusion (Time after restoring of vessel blood flow until occlusion of the vessel indicated by a flow less than 3.0 ml/min)
- Patency (Time during which perfusion of graft is measured related to an observation period of 120 min after administration of test compounds).

Statistical analysis:
Time until occlusion and patency are expressed as median and the interquartile range/2 (IQR/2). Significant differences ($p < 0.05$) are calculated by the non-parametric Kruskal-Wallis test.

CRITICAL ASSESSMENT
The eversion graft is very thrombogenic, although technically difficult and time consuming. The deep occlusive thrombi can prevented only by intraarterially administered thrombolytics or aggressive antithrombotic treatments such as recombinant hirudin at high dosages, or PEG-hirudin. The adventitial surface is a non-endothelial tissue containing tissue-factor and collagen. Thus, both the coagulation system and blood platelets are activated.

MODIFICATIONS OF THE METHOD
Gold et al. (1991) modified the model to be used in thoracotomized dogs in partial obstructed left circumflexed coronary arteries. The combination of reduced blood flow due to the constrictor, along with an abnormal non-endothelial surface, produces total thrombotic occlusion within 5 min.

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B.2.7.3 Arterio-venous shunt thrombosis

PURPOSE AND RATIONALE
A method for the direct observation of extracorporal thrombus formation has been introduced by Rowntree and Shionoya (1927). These first studies could provide evidence that anticoagulants like heparin and hirudin do inhibit thrombus development in arteriovenous shunts. Since today, the A-V-shunt thrombosis models have been often used to evaluate the antithrombotic potential of new compounds in different species including rabbits (Knabb et al. 1992), rats (Hara et al. 1995), pigs (Scott et al. 1994), dogs and cats (Best et al. 1938), and non-human primates (Yokoyama et al. 1995).

PROCEDURE
Rats are anaesthetized and fixed in supine position on a temperature-controlled heating plate to maintain body temperature. The left carotid artery and the right jugular vein are catheterized with short polyethylene catheters. The catheters are filled with isotonic saline solution and clamped. The two ends of the catheters are connected with a 2 cm glass capillary with an internal diameter of 1 mm. This glass capillary provides the thrombogenic surface. At a defined time after administration of the test compound, the clamps which are occluding the A-V-shunt are opened.

The measurement of the patency of the shunt is performed indirectly with a NiCrNi-thermocouple which is fixed distal to the glass capillary. If blood is flowing the temperature rises from room temperature to body temperature. In contrast, decreases of temperature indicates the formation of an occluding thrombus. The temperature is measured continuously over 30 min after opening of the shunt.

CRITICAL ASSESSMENT
It has been shown by Best et al. (1938) that the thrombi formed in the AV-shunt are to a greater part white arterial thrombi. This might be due to the high pressure and shear rate inside the shunts, the thrombi tend to be more arterial in character (Chi et al. 1999).

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B.2.7.4 Thread-induced venous thrombosis

PURPOSE AND RATIONALE

Compared to the arterial system, it seems to be more difficult to develop a thrombosis model in venous blood vessels with respect to reproducibility and variability (Chi et al. 1999). Complete stasis together with a thrombogenic stimulus (Wessler-type) is used by numerous investigators to evaluate the effect of compounds on venous thrombosis. Hollenbach et al. (1994) developed a rabbit model of venous thrombosis by inducingcotton threads into the abdominal vena cava of rabbits. The cotton threads serve as a thrombogenic surface, and a thrombus forms around it growing to a maximum mass after 2–3 h. The prolonged non-occlusive character of thrombogenesis in this model focuses on progression of thrombus formation rather than initiation. Therefore, the conditions more closely resemble pathophysiology in humans because blood continues to flow throughout the experiment (Chi et al. 1999).

PROCEDURE

Rabbits weighing between 2.5 and 3.5 kg are anaesthetized with isofluorane inhalation anesthesia and a polyethylene catheter is inserted into the left carotid artery. A polyethylene tube (PE 240, inner diameter 1.67 mm) of 14 cm length is filled with isotonic saline and a copper wire with 5 fixed cotton threads (length 6 cm) is inserted into the tube (after determination of the net weight of the cotton threads). A laparotomy is performed and the V. cava and V. iliaca are dissected free from surrounded tissue. The test agent is administered by a rabbit intragastric tube 60 min (depending on the *ex vivo* study) prior to initiation of thrombus formation. Blood samples will be measured at 60, 90, 120 and 150 and 210 min after oral administration of the test compound.

Thrombus formation is induced by the inserting the thrombosis catheter into the caval vein via the V. iliaca (7 cm). Then the copper wire is pushed forward 3 cm to liberate the cotton threads into the vessel lumen. 150 min after thrombus initiation, the caval segment containing the cotton threads and the developed thrombus will be removed, longitudinally opened and the content is blotted on filter paper. After weighing the cotton thread with the thrombus, the net thread weight will be subtracted to determine the corrected thrombus weight.

EVALUATION

- Corrected thrombus weight after blotting the thrombus on filter paper and subtraction of the net weight of the cotton thread.
- Mean arterial blood pressure (MAP)
- APTT, HepTest, antiFIIa- and antiFXa-activity

CRITICAL ASSESSMENT

The composition of the cotton threaded thrombus shows a composition of fibrin together with tightly aggregated and distorted erythrocytes thus being in accordance with human deep vein thrombosis structure. Non-occlusive thrombus formation has been successfully inhibited by heparins, prothrombinase complex inhibitors and thrombin inhibitors (Hollenbach et al. 1994, 1995).

MODIFICATIONS OF THE METHOD

In addition to the originally described method, it is possible to measure blood flow by means of a ultrasonic flow probe, attached distally to the position of the cotton threads on the vein.

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B.2.7.5 Thrombus formation on superfused tendon

PURPOSE AND RATIONALE

In all models which include vessel wall damage blood gets in contact with adhesive proteins of the subendothelial matrix, i.e. von Willebrand factor, collagens, fibronectin, laminin and others. Gryglewski et al. (1978) described an *in vivo* method where blood of an unanes-thetized animal is in contact *ex vivo* with a foreign surface consisting mainly of collagen. The foreign surface is produced out of the tendon of another animal species. After superfusion of the tendon, blood is recirculated to the unanesthetized animal. The method aims at the quantitation of the antiplatelet potency of drugs based on the for-mation of platelet thrombi onto the surface of the tendons or of aortic strips from atherosclerotic rabbits.

PROCEDURE

Blood was withdrawn from the carotid artery of anesthetized and heparinized cats by a roller pump at a speed of 6 ml/min. After a passage through a warmed jacket (37 °C), blood was separated into 2 streams, each flowing at a speed of 3 ml/min superfusing in parallel 2 twin strips of the central part of longitudinally cut rabbit Achilles tendon (30 × 3 mm). Instead of rabbit tendons, spirally cut strips from aortas of atherosclerotic rabbits were used. The blood superfusing the strips dripped into collectors and by its gravity was returned to the venous system of the animals through the left jugular vein. The tissue strips were freely suspended in air and the upper end was tied to an auxotonic lever of a smooth muscle/heart Harvard transducer, while the lower end was loaded with a weight (1–2 g) to keep the lever with its counterweight in a neutral position. When superfused with blood, the strips were successively covered with clots changing the weight of the strips. The weight changes were continuously recorded. After a control period of 30 min, the formed thrombi were gently removed and fixed in formalin for histological examination. Then, the strips were superfused with Tyrode solution and the animals injected with the antithrombotic drug. After 10 min, blood superfusion was renewed for another 30 min.

EVALUATION

The ratio of an increase in weight of the strips after the drug treatment to the increase in weight before drug treatment was considered as an index of antiaggregatory activity.

CRITICAL ASSESSMENT

The method described needs two different animal species (rabbit and cat). The reproducibility is not very high. Replacing cats by rabbits was not successful in our hands (Just).

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B.2.8 Stasis-induced thrombosis (Wessler model)

PURPOSE AND RATIONALE

The "Wessler model" is a classical method of inducing venous thrombosis in animals. Wessler (1952, 1953, 1955, 1957, 1959) combined local venous stasis with hypercoagulability produced by injection of human or dog serum into the systemic circulation of dogs or rabbits. The jugular vein of these animals is occluded by clamps 1 min after the injection of the procoagulatory stimulus into the circulation. Within a few min after clamping, a red clot is formed in the isolated venous segment. Fareed et al. (1985) summarized a variety of substances which can be used as pro-coagulatory stimuli. Aronson and Thomas (1985) found an inverse correlation between the duration of stasis and the amount of the hypercoagulating agents to produce the clot.

PROCEDURE

Anaesthetized rabbits are fixed in supine position on a temperature-controlled (37 °C) heating-table. Following cannulation of both carotid arteries (the left in cranial direction) and the right V. femoralis, segments of 2 cm length of the two external jugular veins are exposed and isolated between two loose sutures. 0.3 ml/kg calcium thromboplastin (SIGMA, Deisenhofen, Germany, FRG) is administered via the left carotid artery. Meticulous care is taken to maintain a standard injection time of 30 s followed by injection of 0.5 ml physiological saline within 15 s. 45 s later, both jugular vein segments are occluded by distal and proximal sutures. Stasis is maintained for 30 min. Blood samples are taken immediately before occlusion and 30 s before end of stasis. After excision, the occluded vessel segments are placed on a soaked sponge and opened by a longitudinal incision.

EVALUATION

The size of the clots is assessed using a score system: (0: blood only; 1: very small clot piece(s), filling out at

most 1/4 of the vessel; 2: larger clot piece(s), filling out at most 1/2 of the vessel; 3: very large clot(s), filling out at most 3/4 of the vessel; 4: one large clot, filling out the whole vessel). The scores of the left and the right jugular vein are added forming the thrombus size value of one animal. Additionally, the thrombus weight is measured after blotting the thrombus on filter paper.

Thrombus score is expressed as median (minimum – maximum). Thrombus weight is given as mean ±SEM. For the statistical evaluation of the antithrombotic effect, the nonparametric U-Test of Mann and Whitney (thrombus score) or Student's *t*-test for unpaired samples (thrombus weight) is used. Significance is expressed as $p < 0.05$.

CRITICAL ASSESSMENT

Breddin (1989) described the Wessler model because of its static character as the retransformation of an *in vitro* experiment into a very artificial test situation. One of the major drawbacks is the relative independence of platelet function and hemodynamic changes that largely influence thrombus formation *in vivo*. However, the model has been shown to be very useful for evaluation of the antithrombotic effect of compounds like heparin and hirudin.

MODIFICATIONS OF THE METHOD

There are a number of different procoagulant agents which had been used to induce thrombosis in this model, such as human serum, Russel viper venom, thromboplastin, thrombin, activated prothrombin complex concentrates and factor X_a. (Aronson ant Thomas 1985; Fareed et al. 1985). The sensitivity and accuracy of the model can be improved by injecting iodinated fibrinogen into the animals before injecting the thrombogenic agent and then measuring the specific radioactivity in the clot.

The general drawback of the Wessler model is the static nature of the venous thrombus development. To overcome this problem some investigators have developed more dynamic models with reperfusion of the occluded vessel segments after clot development. Depending on the time of test compound administration (pre- or post-thrombus initiation), the effect on thrombus growth and fibrinolysis can be evaluated. Levi et al. (1992) have used this model to assess the effects of a murine monoclonal anti-human PAI-1 antibody and Biemond et al. (1996) compared the effect of thrombin- and factor X_a-inhibitors with a low molecular weight heparin.

Venous reperfusion model: New Zealand white rabbits weighing 2.5 kg are anesthetized with 0.1 ml atropine, 1.0 mg/kg diazepam, and 0.3 ml Hypnorm (Duphar, 10 mg/ml fluanisone and 0.2 ml fentanyl). Further anesthesia is maintained with 4 mg/kg i.v. thiopental. The carotid artery is cannulated after exposition through an incision in the neck. The jugular vein is dissected free from tissue and small side branches are ligated over a distance of 2 cm. The vein is clamped proximally and distally to isolate the vein segment. Citrated rabbit blood (from another rabbit) is mixed with ^{131}I-radiolabeled fibrinogen (final radioactivity, approximately 25 mCi/ml). 150 µl of this blood is then aspirated in a 1-ml syringe containing 25 µl thrombin (3.75 IU) and 45 µl 0.25 mol $CaCl_2$, and 200 µl of the clotting blood is immediately injected into the isolated segment. 30 min after clot injection the vessel clamps are removed and blood flow is restored. ^{125}I-radiolabeled fibrinogen (approximately 5 µCi) is injected through the cannula in the carotid artery, (in case of the fibrinolysis studies immediately followed by 0.5 mg/kg recombinant tissue-type plasminogen activator). For each dosage group, four thrombi are analyzed. The extent of thrombolysis is assessed by measurement of the remaining ^{131}I-fibrinogen in the clot and compared with the initial clot radioactivity. The comparison between blood and thrombus ^{125}I-radioactivity reveals the extent of thrombus growth (blood volume accreted to the blood). The thrombus lysis and extension are monitored 60 or 120 min after thrombus formation and are expressed as percentage of the initial thrombus volume. Statistics is performed as variance analysis and the Newman-Keuls test. Statistical significance is expressed at the level of $p < 0.05$.

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B.2.9 Experimental thrombocytopenia or leucocytopenia

PURPOSE AND RATIONALE

Intravenous administration of collagen, arachidonic acid, ADP, PAF (platelet activating factor) or thrombin activates thrombocytes leading to a maximal thrombocytopenia within a few minutes. The effect is reinforced by additional injections of epinephrine. Activation of platelets leads to intravasal aggregation and temporary sequestration of aggregates in the lungs and other organs. Depending on the dose of agonist, this experimentally induced reduction of the number of circulating platelets is reversible within 60 min after induction. Following administration of PAF, a leucocytopenia is induced in addition. The assay is used to test the inhibitory capacity of drugs against thrombocytopenia or leucocytopenia as a consequence of *in vivo* platelet or leukocyte stimulation.

PROCEDURE

Materials and solutions

Substances used to induce thrombocytopenia/ leucocytopenia (intravenous administration)

In rabbits:		
arachidonic acid (Sigma)	1	mg/kg
collagen (Hormonchemie)	30	µg/ml
In mice:		
collagen	90	µg/kg
+ adrenaline (Hormonchemie)	+ 20	µg/kg
In hamsters:		
collagen	50	µg/kg
+ adrenaline	+ 10	µg/kg
In guinea pigs:		
PAF (Paf-acether, Bachem)	0.03–0.04	µg/kg
thrombin (Hoffman-LaRoche)	60	U/kg
Anesthetics:		
pentobarbital sodium (i.p.)	30	mg/kg
xylazine (i.m.)	8	mg/kg
urethane (i.p.)	1.5	g/kg

Platelet analyzer: Sysmex microcellcounter F-800

Male guinea pigs (Pirbright White) weighing 300–600 g, or male NMRI mice (25–36 g), or Chinchilla rabbits of either sex weighing 2–3 kg are used. Animals receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (p.o. 60 min, i.p. 30 min, i.v. variable), the marginal vein of the ear of rabbits is cannulated and the thrombocytopenia-inducing substances collagen or arachidonic acid are injected slowly. Blood is collected from the ear artery.

Guinea pigs, hamsters, or mice are anesthetized with pentobarbital sodium (i.p.) and Rompun® (i.m.) and placed on an electrically warmed table at 37 °C. The carotid artery is cannulated for blood withdrawal and the jugular vein is cannulated to administer the thrombocytopenia-inducing substances collagen + adrenaline (injection of the mixture of both within 10 s) or PAF or thrombin. In mice collagen + adrenaline are injected into a tail vein.

Approx. 50–100 µl blood are collected into potassium-EDTA-coated tubes at times –1, 1 and 2 min (guinea pigs and mice) or 5, 10 and 15 min (rabbits) following the injection of the inducer. The number of platelets and leukocytes is determined within 1 h after withdrawal in 10 µl samples of whole blood using a microcellcounter suitable for blood of various animal species.

EVALUATION

The percentage of thrombocytes (or leukocytes) is determined in vehicle control and dosage groups at the different times following injection of the inducer relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are taken as 100%.

Percent inhibition of thrombocytopenia (or leucocytopenia) is calculated in dosage groups relative to controls.

Statistical significance is evaluated by means of the unpaired Student's *t*-test.

CRITICAL ASSESSMENT

The method of collagen + epinephrine induced thrombocytopenia is presently widely used to study the phenotype of mice knocked out for a specific gene with suspected role in hemostasis /thrombosis. A recent example is the Gas 6 –/– mouse (Angelillo-Scherrer et al. 2001) and mice lacking the gene for the G protein G(z) (Yang et al. 2000). The advantage of the method for this purpose is the simple experimental procedure and the small volume of blood necessary. In general, application of the method in small animals (mice, hamsters) needs only small amounts of drug substance. The model is a useful first step of *in vivo* antithrombotic efficacy of antiplatelet drugs.

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B.2.10 Collagenase-induced thrombocytopenia

PURPOSE AND RATIONALE

Intravenous administration of the proteolytic enzyme collagenase leads to formation of endothelial gaps and to exposure of deeper layers of the vessel wall. This vascular endothelial injury is mainly involved in triggering thrombus formation by activation of platelets through contact with the basal lamina. As a consequence, thrombocytopenia is induced, which is maximal within 5–10 min following collagenase injection and reversible within 30 min after induction. The model is used to test the inhibitory capacity of compounds against thrombocytopenia in a model of collagenase-induced thrombocytopenia in rats as an alternative to the model described before

PROCEDURE

Materials and solutions

Anesthetic: pentobarbital sodium (i.p.)	60	mg/kg
Heparin (Liquemin®) (i.v.)	500	U/kg
To induce thrombocytopenia (intravenous administration): collagenase (E.C. 3.4.24.3), (Boehringer, Mannheim)	10	mg/ml/kg
Platelet analyzer: Sysmex microcellcounter F-800		

Male Sprague-Dawley rats weighing 260–300 g are used. The animals receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), rats are anesthetized with pentobarbital sodium (i.p.). One carotid artery is cannulated for blood withdrawal and one jugular vein is cannulated for inducer injection. The animals receive an intravenous injection of heparin and 20 min later, approx. 100 µl blood are collected (initial value). Ten min later, the thrombocytopenia-inducing substance collagenase is administered intravenously.

At times 5, 10, 20 and 30 min following the injection of collagenase, samples of approx. 100 µl blood are collected into potassium-EDTA-coated tubes. The number of platelets is determined in 10 µl samples of whole blood within 1 h after blood withdrawal using a microcellcounter.

EVALUATION

1. The percentage of platelets is determined in vehicle control and dosage groups at the different times following injection of collagenase relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are set 100%.
2. Percent inhibition of thrombocytopenia is calculated in dosage groups relative to controls.

Statistical significance is evaluated by means of the unpaired Student's *t*-test.

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B.2.11 Reversible intravital aggregation of platelets

PURPOSE AND RATIONALE

Isotopic labeling of platelets can be employed to monitor platelet aggregation and desegregation *in vivo*. ADP, (adenosine diphosphate), PAF (platelet activating factor), arachidonic acid, thrombin and collagen are known to induce platelet aggregation. In the following procedure, labelled platelets are continuously monitored in the thoracic (A) and abdominal (B) region of test animals. Administration of aggregation promoting agents produces an increase in counts in A and a fall in counts in B. This observation implies that platelets are being aggregated within the vascular system and accumulate in the pulmonary microvasculature. The *in-vivo* method can be used to evaluate platelet anti-aggregatory properties of test compounds.

PROCEDURE

Preparation of labelled platelets

Blood is obtained from rats by cardiopuncture. After centrifugation at 240 *g* for 10 min, the platelet rich plasma (PRP) is transferred into a tube and suspended in calcium free Tyrode solution containing 250 ng/ml PGE_1. The suspension is centrifuged at 640 *g* for 10 min. The supernatant is discarded and the sediment is suspended by gentle shaking with calcium free Tyrode solution containing 250 ng/ml PGE_1. ^{51}Cr is added to 1 ml of the platelet suspension. Following a 20 min incubation period at 37 °C, the suspension is again centrifuged at 640 *g* for 10 min. The supernatant is removed and the labelled platelets are finally resuspended in 1 ml calcium free Tyrode solution containing 250 ng/ml PGE_1.

In vivo experiment

Male Sprague-Dawley or stroke-prone spontaneously hypertensive rats weighing 150–300 g are used. The animals are anaesthetized with pentobarbital sodium (30 mg/kg, i.p.). Following tracheotomy, the vena femoralis is exposed and cannulated. The labelled platelets are administered via the cannula. The circulating platelets are monitored continuously in the thoracic (A) and abdominal (B) region. The counts are collected using a dual channel gamma spectrometer (Nuclear Enterprise 4681) incorporating a microcomputer (AM 9080A). One h after administration of labelled platelets (when counts in A and B have stabilized), the aggregation promoting agent (ADP, PAF, arachidonic acid, thrombin or collagen) is administered twice by intravenous injection. One hour is allowed to elapse between each i.v. injection.

The test compound is administered 2 h after platelet injection concurrently with the fourth administration of the aggregating agent. Thirty minutes (ADP, PAF, arachidonic acid, thrombin), or 1 h (collagen) after compound administration another control injection of the aggregating agent is given. This injection is either used as an additional control or it may reveal long-term efficacy of a test compound.

Standard compound:

- PGI_2 (prostacyclin)

EVALUATION

The microcomputer continuously reveals information about aggregation and desegregation of labelled platelets.

The following parameters are recorded

- A = counts over thorax
- B = counts over abdomen
- Difference: $A - B$
- ratio: A / B

The time course of response is shown in a curve. The area under the curve is calculated by a computer program.

Statistical significance is calculated using the Student's *t*-test.

MODIFICATION OF THE METHOD

Oyekan and Botting (1986) described a method for monitoring platelet aggregation *in vivo* in rats, using platelets labeled with indium^{3+} oxine and recording the increase in radioactivity count in the lung after injection of adenosine diphosphate or collagen.

Smith et al. (1989) monitored continuously the intrathoracic content of intravenously injected ^{111}indium labeled platelets in anesthetized guinea pigs using a microcomputer-based system.

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B.3
Bleeding models

B.3.1
Subaqueous tail bleeding time in rodents

PURPOSE AND RATIONALE

The damage of a blood vessel results in the formation of a hemostatic plug, which is achieved by several different mechanisms including vascular spasm, formation of a platelet plug, blood coagulation, and growth of fibrous tissue into the blood clot.

A diagnostic parameter for specific defects of the hemostatic system and for the influence of drugs affecting hemostasis is the length of time that it takes for bleeding to stop from a standard incision, the so-called bleeding time.

Bleeding-time measurements in animals are used to evaluate the hemorrhagic properties of antithrombotic drugs. The transection of the tail of a rodent was first established by Döttl and Ripke (1936) and is commonly used in experimental pharmacology.

PROCEDURE

Anaesthetized rats are fixed in supine position on a temperature-controlled (37 °C) heating-table. Following catheterization of a carotid artery (for measurement of blood pressure) and a jugular vein, the test compound is administered. After a defined latency period, the tail of the rat is transected with a razor-blade mounted on a self-constructed device at a distance of 4 mm from the tip of the tail. Immediately after transection, the tail is immersed into a bath filled with isotonic saline solution (37 °C).

EVALUATION

The time until bleeding stops is determined within a maximum observation time of 600 s.

CRITICAL ASSESSMENT OF THE METHOD

There are numerous variables which can influence rodent's bleeding time measurements as discussed by Dejana et al. (1982): position of the tail (horizontal or vertical), the environment (air or saline), temperature, anesthesia, procedure of injury (Simplate method, transsection). All these variables are responsible for the different results reported in literature on compounds like aspirin and heparin under different assay condition (Stella et al. 1975; Minsker and Kling 1977).

Furthermore, it is impossible to transect exactly one blood vessel, because the transected tail region consists of a few major arteries and veins with mutual interaction between one another.

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B.3.2 Arterial bleeding time in mesentery

PURPOSE AND RATIONALE

Arterial bleeding is induced by micropuncture of small arteries in the area supplied by the mesenteric artery. Bleeding is arrested in living blood vessels by the formation of a hemostatic plug due to the aggregation of platelets and to fibrin formation. In this test, compounds are evaluated which inhibit thrombus formation thus prolonging arterial bleeding time. The test is used to detect agents which interfere with primary hemostasis in small arteries.

PROCEDURE

Male Sprague-Dawley rats weighing 180–240 g receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), the animals are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. Rats are placed on an electrically warmed table at 37 °C.

The abdomen is opened by a mid-line incision and the mesentery is lifted to display the mesenteric arteries. The mesentery is draped over a plastic plate and superfused continuously with Tyrode's solution maintained at 37 °C. Bleeding times are determined with small mesenteric arteries (125–250 µm external diameter) at the junction of mesentery with intestines. Adipose tissue surrounding the vessels is carefully cut with a surgical blade.

Arteries are punctured with a hypodermic needle (25 gauge: 16 × 5/10 mm). The bleeding time of the mesenteric blood vessels is observed through a microscope at a magnification of 40×. The time in seconds is determined from the puncturing until the bleeding is arrested by a hemostatic plug.

EVALUATION

1. Mean values of bleeding times [s] are determined for each dosage group (4–6 animals, 4–6 punctures each) and compared to the controls.

   The significance of the results is assessed with the unpaired Student's *t*-test.
2. The percent prolongation of bleeding time in dosage groups relative to the vehicle controls is calculated.

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B.3.3 Template bleeding time method

PURPOSE AND RATIONALE

The template bleeding time methods is used to produce a standardized linear incision into the skin of humans to detect abnormalities of primary hemostasis due to deficiencies in the platelet or coagulation system. The method has been modified with the develop-

ment of a spring-loaded cassette with two disposable blades (Simplate II, Organon Teknika, Durham, NC). These template devices ensure reproducibility of length and depth of dermal incisions. Forsythe and Willis (1989) described a method which enables the Simplate technique as a method to analyze the bleeding time in the oral mucosa of dogs.

PROCEDURE

The dog is positioned in sternal or lateral recumbency. A strip of gauze is tied around both the mandible and maxilla as a muzzle. The template device is placed evenly against the buccal mucosa, parallel to the lip margin, and triggered. Simultaneously, a stopwatch is started. Blood flow from the incision is blotted using circular filter paper (Whatman No. 1, Fisher Scientific Co., Clifton, NJ) held directly below, but not touching the wounds. The position of the filter paper is changed every 15 s. The end point for each bleeding is determined when the filter paper no longer develops a red crescent.

EVALUATION

The time from triggering the device until blood no longer appears on the paper is recorded as the bleeding time. The normal range lies between two to four minutes.

CRITICAL ASSESSMENT OF THE METHOD

The template bleeding time varies considerably between laboratories as well as between species and strains. Therefore, it is important to perform the incisions and the blotting in an identical fashion. Prolonged bleeding times in dogs have been recognized with thrombocytopenia, von Willebrand's disease, uremia, treatment with aspirin, anticoagulants, and dextran (Forsythe and Willis 1989; Klement et al. 1998). Brassard and Meyers (1991) describe the buccal mucosa bleeding time as a test which is sensitive to platelet adhesion and aggregation deficits. Generally, results of antithrombotic drugs in bleeding time models in animals do not exactly predict bleeding risks in clinical situations. But the models allow comparison between drugs with different actions (Dejana et al. 1979; Lind et al. 1991).

MODIFICATIONS OF THE METHOD

The Simplate device can also be used to perform incisions at the shaved inner ear of rabbits taking care to avoid major vessels. The normal range of bleeding time in anaesthetized rabbits is approximately 100 s (77 ±4 s, $n = 20$) in our own laboratory (Just et al. unpublished).

Klement et al. (1998) described another ear bleeding model in anaesthetized rabbits. The shaved ear was immersed in a beaker containing saline at 37 °C. Five full-thickness cuts were made with a no. 11 Bard-Parker scalpel blade avoiding major vessels and the ear was immediately re-immersed in saline. At different times thereafter (5 to 30 min) aliquots of the saline solution were removed, red cells were sedimented and lysed, and cyanohemoglobin was determined as a measure of blood loss. In this study, hirudin produced more bleeding than standard heparin.

A cuticle bleeding time (toenail bleeding time) measurement in dogs has been described by Giles et al. (1982). A guillotine type toenail clipper is used to sever the apex of the nail cuticle. A clean transection of the nail is made just into the quick, to produce a free flow of blood. The nail is left to bleed freely. The time until bleeding stops is recorded as the bleeding time. Several nails can be cut at one time to ensure appropriate technique. The normal range lies between two to eight minutes.

REFERENCES

Brassard JA and Meyers KM (1991) Evaluation of the buccal bleeding time and platelet glass bead retention as assays of hemostasis in the dog: the effects of acetylsalicylic acid, warfarin and von Willebrand factor deficiency. Thromb Haemost 65:191–195

Dejana E, Calloni A, Quintana A, deGaetano G (1979) Bleeding time in laboratory animals. II A comparison of different assay conditions in rats. Thromb Res 15:191–197

Forsythe LT and Willis SE (1989) Evaluating oral mucosa bleeding times in healthy dogs using a spring loaded device. Can Vet J 30:344–345

Giles AR, Tinlin S and Greenwood R (1982) A canine model of hemophilic (factor VIII:C deficiency) bleeding. Blood 60: 727–730

Klement P, Liao P, Hirsh J, Jonston M, Weitz JI (1998) Hirudin causes more bleeding than heparin in a rabbit ear bleeding model. J Lab Clin Med 132:181–185

Lind SE (1991) The bleeding time does not predict surgical bleeding. Blood 77:2547–2552

B.4 Genetically modified animals

B.4.1 Knock out mice

PURPOSE AND RATIONALE

Genetically modified animals, in particular knock-out mice, help to understand the role of various factors in blood clotting, thrombolysis, and platelet function. They are useful to verify the mode of action of new drugs.

Factor I (Fibrinogen)

Phenotype

Born in normal appearance, ~10% die shortly after birth and another 40% around 1–2 months after birth due to bleeding, failure of pregnancy, blood samples fail to clot or support platelet aggregation *in vitro*.

References
Suh TT, Holmback K, Jensen NJ, Daugherty CC, Small K, Simon DI, Potter SS, Degen JL (1995) Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev 9:2020–2033

Factor II (Prothrombin)

Phenotype
Partial embryonic lethality: 50% between embryonic day (E) 9.5–11.5; at least 1/4 survive to term, but fatal hemorrhage few days after birth; factor II important in maintaining vascular integrity during development as well as postnatal life.

References
Sun WY, Witte DP, Degen JL, Colbert MC, Burkart MC, Holmbäck K, Xiao Q, Bugge TH, Degen SJF (1998) Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc Natl Acad Sci USA 95:7597–7602
Xu J, Wu Q, Westfield L, Tuley EA, Lu D, Zhang Q, Shim K, Zheng X, Sadler JE (1998) Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. Proc Natl Acad Sci USA 95:7603–7607

Factor V

Phenotype
Half of the embryos die at E9–10, possibly as a result of abnormal yolk sac vasculature, remaining 50% progress normally to term, but die from massive hemorrhage within 2 h of birth, more severe in mouse than in human (due to residual activity in humans).

References
Cui J, O'Shea KS, Purkayastha A, Saunders TL, Ginsburg D (1996) Fatal hemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature 384: 66–68
Yang TL, Cui J, Taylor JM, Yang A, Gruber SB. Ginsburg D (2000) Rescue of fatal neonatal hemorrhage in factor V deficient mice by low level transgene expression. Thromb Haemost 83:70–77

Factor VII

Phenotype
Develop normally but suffer fatal perinatal bleeding.

References
Rosen ED, Chan JCY, Idusogie E, Clotman F, Vlasuk G, Luther T, Jalbert LR, Albrecht S, Zhong L, Lissens A, Schoonjans L, Moons L, Collen D, Castellino FJ, Carmeliet P (1997) Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. Nature 390:290–294

Factor VIII

Phenotype
Mild phenotype compared with severe hemophilia A in humans; no spontaneous bleeding, illness or reduced activity during the first year of life; have residual clotting activity (APTT).

References
Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazaazian HH (1995) Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet 10: 119–121

Factor IX

Phenotype
Factor IX coagulant activities (APTT): +/+ 92%, +/– 53%, –/– <5%; bleeding disorder (extensive bleeding after clipping a portion of the tail, bleeding to death if not cauterized.

References
Kundu RK, Sangiorgi F, Wu LY, Karachi K, Anderson WF, Maxson R, Gordon EM (1998) Targeted inactivation of the coagulation factor IX gene causes hemophilia B in mice. Blood 92: 168–174
Wang L, Zoppe M, Hackeng TM, Griffin JH, Lee K-F, Verma IM (1997) A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci USA 94:11563–11566

Factor X

Phenotype
Partial embryonic lethality (1/3 died on E11.5-12.5); fatal neonatal bleeding between postnatal day (P) 5-20.

References
Dewerchin M,, Liang Z, Moons L, Carmeliet P, Castellino FJ, Collen D, Rosen ED (2000) Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost 83:185–190

Factor XI

Phenotype
APTT prolonged in –/– (158–200 s) compared with +/+ (25–34 s) and +/– (40–61 s); no factor XI activity and antigen, did not result in intrauterine death, –/– similar bleeding as +/+ with a tendency to prolongation.

References
Gailani D, Lasky NM, Broze GJ (1997) A murine model of factor XI deficiency. Blood Coagul Fibrinolysis 8:134–144

Factor XII
Not described

Factor XIII
Not described

TF (tissue factor)

Phenotype
Abnormal circulation from yolk sac to embryo ~E8.5 leading to embryo wasting and death; TF has a role in blood vessel development.

References
Bugge TH, Xiao Q, Kombrinck KW, Flick MJ, Holmback K (1996) Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci U S A 93:6258–6263

Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlanderen I, Demunck H, Kasper M, Breier G, Evrard P, Müller M, Risau W, Edgington T and Collen D (1996) Role of tissue factor in embryonic blood vessel development. Nature 383:73–75

Toomey JR, Kratzer KE, Lasky NM, Stanton JL, Broze GJ (1996) Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood 88:1583–1587

Toomey JR, Kratzer KE, Lasky NM, Broze GJ (1997) Effect of tissue factor deficiency on mouse and tumor development. Proc Natl Acad Sci USA 94:6922–6926

TFPI (tissue factor pathway inhibitor)

Phenotype
None survive the neonatal period; 60% die between E9.5–11.5 with signs of yolk sac hemorrhage.

References
Huang ZF, Higuchi D, Lasky N, Broze GJ (1997) Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 90:944–951

Thrombin receptor

Phenotype
50% die at E9–10; 50% survive and become grossly normal adult mice with no bleeding diathesis; –/– platelets strongly respond to thrombin; –/– fibroblast loose their ability to respond to thrombin → second TR must exist.

References
Connolly AJ, Ishihara H, Kahn ML, Farese RV Jr., Coughlin SR (1996) Role of the thrombin receptor in development and evidence for a second receptor. Nature 381:516–519

Darrow AL, Fung-Leung WP, YE RD, Santulli RJ, Cheung WM, Derian CK, Burns CL, Damiano BP, Zhou L, Keenan CM, Peterson PA, Andrade-Gordon P (1996) Biological consequences of thrombin receptor deficiency in mice. Thromb Haemost 76: 860–866

Thrombomodulin

Phenotype
Embryonic lethality before development of a functional cardiovascular system; die before E9.5 due to retardation of growth; TM+/– mice develop normal without thrombotic complications.

References
Christie PD, Edelberg JM, Picard MH, Foulkes AS, Mamuya W (1999) A murine model of myocardial microvascular thrombosis. J Clin Invest 104:533–539

Healy AM, Rayburn HB, Rosenberg RD, Weiler H (1995) Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc Natl Acad Sci USA 92:850–854

Healy AM, Hancock WW, Christie PD, Rayburn HB, Rosenberg RD (1998) Intravascular coagulation activation in a murine model of thrombomodulin deficiency: effects of lesion size, age, and hypoxia on fibrin deposition. Blood 92:4188–4197

Weiler-Guettler H, Christie PD, Beeler DL, Healy AM, Hancock WW (1998) A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest 101:1983–1991

Protein C

Phenotype
KO mice appeared to develop normally macroscopically, but possessed obvious signs of bleeding and thrombosis; did not survive beyond 24 h after delivery; microvascular thrombosis in the brain and necrosis in the liver; plasma clottable fibrinogen was not detectable suggesting fibrinogen depletion and secondary consumptive coagulopathy.

References
Jalbert LR, Rosen ED, Moons L, Chan JCY, Carmeliet P (1998) Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest 102:1481–1488

Protein S
Not described

Plasminogen

Phenotype
Severe spontaneous thrombosis; reduced ovulation and fertility; cachexia and shorter survival; severe glomerulonephritis; impaired skin healing; reduced macrophage and keratinocyte migration.

References
Bugge TH, Flick MJ, Daugherty CC, Degen JL (1995) Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. Genes Dev 9:794–807

Ploplis VA, Carmeliet P, Vazirzadeh S, Van Vlaenderen I, Moons L (1995) Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. Circulation 92:2585–2593

Alpha$_2$-antiplasmin

Phenotype

Normal fertility, viability and development; no bleeding disorder; spontaneous lysis of injected clots → enhanced fibrinolytic potential; significant reduction of renal fibrin deposition after LPS.

References

Lijnen HR, Okada K, Matsuo O Collen D, Dewerchin M (1999) alpha$_2$-antiplasmin gene deficiency in mice is associated with enhanced fibrinolytic potential without overt bleeding. Blood 93:2274–2281

t-PA (tissue-type plasminogen activator)

Phenotype

Extensive spontaneous fibrin deposition; severe spontaneous thrombosis; impaired neointima formation; reduced ovulation and fertility; cachexia and shorter survival; severe glomerulonephritis; abnormal tissue remodelling.

References

Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J (1998) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Christie PD, Edelberg JM, Picard MH, Foulkes AS, Mamuya W (1999) A murine model of myocardial microvascular thrombosis. J Clin Invest 104:533–539

PAI-1 (plasminogen activator inhibitor 1)

Phenotype

Reduced thrombotic incidence; no bleeding; accelerated neo-intima formation; reduced lung inflammation; reduced atherosclerosis.

References

Carmeliet P, Kieckens L, Schoonjans L, Ream B, van Nuffelen NA, Prendergast G, Cole M, Bronson R, Collen D (1993a) Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. J Clin Invest 92:2746–2755

Carmeliet P, Stassen JM, Schoonjans L, Ream B, van den Oord JJ, de Mol M, Mulligan RC, Collen D (1993b) Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemo-stasis, thrombosis, and thrombolysis. J Clin Invest 92:2756–2760

Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D (1996) Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. J Clin Invest 97:232–237

Erickson LA, Fici GJ, Lund JE, Boyle TP, Polites HG, Marotti KR (1990) Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene. Nature 346: 74–76

Kawasaki T, Dewerchin M, Lijnen HR, Vermylen J, Hoylaerts MF (2000) Vascular release of plasminogen activator inhibitor-1 impairs fibrinolysis during acute arterial thrombosis in mice. Blood 96:153–60

Pinsky DJ, Liao H, Lawson CA, Yan SF, Chen J (1998) Coordinated induction of plasminogen activator inhibitor-1 (PAI-1) and inhibition of plasminogen activator gene expression by hypoxia promotes pulmonary vascular fibrin deposition. J Clin Invest 102:919–928

TAFI (thrombin activatable fibrinolysis inhibitor)

Not described

Vitronectin

Phenotype

Normal development, fertility, and survival; serum is completely deficient in "serum spreading factor" and plasminogen activator inhibitor 1 binding activities; delayed arterial and venous thrombus formation.

References

Eitzman DT, Westrick RJ, Nabel EG, Ginsburg D (2000) Plasminogenactivator inhibitor-1 and vitronectin promote vascular thrombosis in mice. Blood 95:577–580

Zheng X, Sunders TL, Camper SA, Samuelson LC, Ginsburg D (1995) Vitronectin is not essential for normal mammalian development and fertility. Proc Natl Acad Sci USA 92: 12426–12430

Urokinase, u-PA (urinary-type plasminogen activator)

Phenotype

Single u-PA deficiency: viable, fertile, normal life span; occasionally spontaneous fibrin deposits in normal and inflamed tissue; higher incidence of endotoxin-induced thrombosis. Combined t-PA and u-PA deficiency: mice survive embryonic development; retarded growth, reduced fertility, shortened life span; spontaneous fibrin deposits more extensively and in more organs.

Transgenic mice carrying the u-PA gene linked to the albumin enhancer/promoter exhibit spontaneous intestinal and intraabdominal bleeding directly related to transgene expression in the liver and elevated plasma u-PA levels. 50% die between 3 and 84 h after birth; severe hypofibrinogenemia, loss of clotting function.

References

Carmeliet P, Schoonjans L, Kieckens L, Ream B; Degen J (1998) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Heckel JL, Sandgren EP, Degen JL, Palmiter RD, Brinster RL (1990) Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. Cell 62:447–456

UPAR (urinary-type plasminogen activator receptor)

Phenotype

Phenotype normal; attenuated thrombocytopenia and mortality associated with severe malaria.

References

Bugge TH, Suh TT, Flick MJ, Daugherty CC, Romer J, Solberg H, Ellis V, Dano K, Degen JL (1995) The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. J Biol Chem 270:16886–16894

Bugge TH, Flick MJ, Danton MJ, Daugherty CC, Romer J, Dano K, Carmeliet P, Collen D, Degen JL (1996) Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. Proc Natl Acad Sci USA 93:5899–5904

Dewerchin M, van Nuffelen A, Wallays G, Bouché, Moons L, Carmeliet P, Mulligan RCM, Collen D (1996) Generation and characterization of urokinase receptor deficient mice. J Clin Invest 97:870–878

Piguet PF, Da-Laperrousaz C, Vesin C, Tacchini-Cottier F, Senaldi G, Grau GE (2000) Delayed mortality and attenuated thrombocytopenia associated with severe malaria in urokinase- and urokinase receptor-deficient mice. Infect Immun 68:3822–3829

Gas 6 (growth arrest-specific gene 6 product)

Phenotype

Mice are viable, fertile, appear normal; do not suffer spontaneous bleeding or thrombosis; have normal tail bleeding time. Platelets fail to aggregate irreversibly to ADP, collagen, or U 46619. Arterial and venous thrombosis is inhibited and mice are protected from fatal thromboembolism after injection of collagen plus epinephrine.

References

Angelillo-Scherrer A, DeFrutos PG, Aparicio C, Melis E, Savi P, Lupu F, Dewerchin M, Hoylaerts MF, Herbert J-M, Collen D, Dahlbaeck B, Carmeliet P (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med 7:215–221

GPIbalpha (glycoprotein Ib alpha, part of the GP Ib-V-IX complex)

Phenotype

Bleeding, thrombocytopenia and giant platelets (similar to human Bernard Soulier syndrome).

References

Ware J, Russell S, Ruggeri ZM (2000) Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. Proc Natl Acad Sci USA 97:2803–2808

GPV (glycoprotein V, part of the GP Ib-V-IX complex)

Phenotype

Increased thrombin responsiveness, GpV–/– platelets are normal in size, normal amounts in GpIb-IX, functional in vWF-binding; platelets are hyperresponsive to thrombin → increased aggregation response; shorter bleeding time; → GpV = negative modulator of platelet function.

References

Ramakrishnan V, Reeves PS, DeGuzman F, Deshpande U, Ministri-Madrid K (1999) Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. Proc Natl Acad Sci USA 96:13336–13341

GPIIb (integrin alpha IIb, glycoprotein IIb, part of the GP IIb-IIIa complex

Phenotype

Bleeding disorder similar to Glanzmann thrombasthenia in man; platelets failed to bind fibrinogen, to aggregate and to retract a fibrinogen clot; α-granules do not contain fibrinogen.

References

Tronik-Le Roux D, Roullot V, Poujol C, Kortulewski T, Nurden P, Marguerie G (2000) Thrombasthenic mice generated by replacement of the integrin alpha$_{IIb}$ gene: demonstration that transcriptional activation of this megakaryocytic locus precedes lineage commitment. Blood 96:1399–1408

GP IIIa (integrin beta3, glycoprotein IIIa, part of the GP IIb-IIIa complex

Phenotype

Viable, fertile, increased fetal mortality; features of Glanzmann thrombasthenia in man, e.g. defective platelet aggregation, clot retraction; spontaneous bleeding, prolonged bleeding times; dysfunctional osteoclasts, development of osteosclerosis with age.

References

Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D (1999) Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 103:229–238

McHugh KP, Hodivala-Dilke K, Zheng M-H, Namba N, Lam J (2000) Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest 105:433–440

GP IIa (glycoprotein IIa, integrin beta 1, part of the GP Ia-IIa complex)

Phenotype

Integrin beta1 null platelets from conditional knockout mice develop normally, platelet count is normal.

Collagen induced platelet aggregation is delayed but otherwise normal; tyrosine phosphorylation pattern is normal but phosphorylation is delayed. Bleeding time in bone marrow chimeric mice is normal; no major *in vivo* defects.

References

NieswandtB, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokhtari-Nejad R, Lindhout T, Heemskerk JWM, Zirngibl H, Faessler R (2001) Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. EMBO J 20:2120–2130

VWF (von Willebrand factor)

Phenotype

Factor VIII levels strongly reduced due to defective protection by vWF; highly prolonged bleeding time, hemorrhage, spontaneous bleeding; mice useful for investigating the role of vWF; delayed platelet adhesion in ferric-chloride-induced arteriolar injury.

References

Denis C, Methia N, Frenette PS, Rayburn M, Ullman-Cullere M (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 95:952–959

Ni H, Denis CV, Subbarao S, Degen JL, Sato TN, Hynes RO, Wagner DD (2000) Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. J Clin Invest 106:385–392

Thromboxane A2 receptor (TXA2r)

Phenotype

Mild bleeding disorder and altered vascular responses to TXA2 and arachidonic acid.

References

Thomas DW, Mannon RB, Mannon PJ, Latour A, Oliver JA (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. J Clin Invest 102:1994–2001

Prostacyclin receptor (PGI2r)

Phenotype

Viable, fertile, normotensive; increased susceptibility to thrombosis; reduced inflammatory and pain responses.

References

Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A (1997) Altered pain reception and inflammatory response in mice lacking prostacyclin receptor. Nature 388:678–682

PECAM (platelet:endothelial cell adhesion molecule)

Phenotype

Normal platelet aggregation; prolonged bleeding time.

References

Duncan GS, Andrew DP, Takimoto H, Kaufman SA, Yoshida H (1999) Genetic evidence for functional redundancy of platelet/endothelial cell adhesion molecule-1 (PECAM-1) CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. J Immunol 162:3022–3030

Mahooti S, Graesser D, Patil S, Newman P, Duncan G, Mak T, Madri JA (2000) PECAM-1 (CD 31) expression modulates bleeding tome *in vivo*. Am J Pathol 157:75–81

Pallid (Pa)

Phenotype

Among 13 hypopigment mouse mutants with storage pool deficiency, the pallid mouse is a model of the human Hermansky Pudlak syndrome (the beige mouse is a model of the Chediak Higashi syndrome). Pallid mice exhibit prolonged bleeding time, pigment dilution, kidney lysosomal enzyme elevation serum alpha 1 antitrypsin deficiency and abnormal otolith formation. The gene defective in pallid mice encodes the highly charged 172-amino acid protein pallidin which interacts with syntaxin 13, a protein mediating vesicle docking and fusion.

References

Huang L, Kuo YM, Gitschier J (1999) The pallid gene encodes a novel, syntaxin 13-interacting protein involved in platelet storage pool deficiency. Nat Genet 23:329–332

G alpha(q) (guanyl nucleotide binding protein G alpha q)

Phenotype

Defective aggregation in response ADP, TXA2, thrombin, collagen; shape change normal.

References

Offermans S, Toombs CF, Hu YH, Simon MI (1997) Defective platelet activation in G alpha(q)-deficient mice. Nature 389:183–186

Ohlmann P, Eckly A, Freund M, Cazenave JP, Offermanns S, Gachet C (2000) ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. Blood 96:2134–2139

G z (member of the Gi family of G proteins)

Phenotype

Impaired platelet aggregation to epinephrine; resistance to fatal thromboembolism; exaggerated response

to cocaine, reduced effect of morphine and antidepressant drugs.

Reference

Yang J, Wu J, Kowalska MA, Dalvi A, Prevost N, O'Brien PJ, Manning D, Poncz M, Lucki I, Blendy JA, Brass LF (2000) Loss of signaling through G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. Proc Natl Acad Sci USA 97:9984–9989

Phospholipase C gamma

Phenotype

Viable, fertile, decreased mature B cells; defective B cell and mast cell function; defective Fc_{gamma} receptor signaling, therefore, loss of collagen induced platelet aggregation.

References

Wang D, Feng J, Wen R, Marine JC, Sangster MY, Parganas E, Hoffmeyer A, Jackson CW, Cleveland JL, Murray PJ, Ihle JN (2000) Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity 13:25–35

CD39 (vascular adenosine triphosphate diphosphohydrolase)

Phenotype

Viable, fertile; prolonged bleeding times but minimally perturbed coagulation parameters; reduced platelet interaction with injured mesenteric vasculature *in vivo*. Platelets fail to aggregate to standard agonists *in vitro* associated with purinergic P2Y1 receptor desensitization; fibrin deposition at multiple organ sites.

References

Enjyoji K, Sevigny J, Lin Y, Frenette PS, Christie PD (1999) Targe-ted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med 5: 1010–1017

Protein kinase, cGMP-dependent, type 1

Phenotype

Viable, fertile; unresponsive to cGMP and NO; defective VASP-phosphorylation; increased adhesion and aggregation of platelets *in vivo* in ischemic /reperfused mesenteric microcirculation; no compensation by cAMP kinase system.

References

Massberg S, Sausbier M, Klatt P, Bauer M, Pfeifer A, Siess W, Faessler R, Ruth P, Krombach F, Hofmann F (1999) Increased adhesion and aggregation of platelets lacking cyclic guanosine 3',5'-monophosphate kinase I. J Exp Med 189:1255–1264

Vasodilator-stimulated phosphoprotein (VASP)

Phenotype

Viable, fertile; mild platelet dysfunction with megakaryocyte hyperplasia, increased collagen /thrombin activation, impaired cyclic nucleotide mediated inhibition of platelet activation.

References

Aszodi A, Pfeifer A, Ahmad M, Glauner M, Zhou XH, Ny L, Andersson KF, Kehrel B, Offermanns S, Faessler R (1999) The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. EMVBO J 18:37–48

Hauser W, Knobeloch KP, Eigenthaler M, Gambaryan S, Krenn V (1999) Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. Proc Natl Acad Sci USA 96:8120–8125

Arachidonate 12-lipoxygenase (P-12LO)

Phenotype

Platelets exhibit a selective hypersensitivity to ADP, manifested as a marked increase in slope and percent aggregation in *ex vivo* assays and increased mortality in an ADP-induced mouse model of thromboembolism.

References

Johnson EN, Brass LF, Funk CD (1998) Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. Proc Natl Acad Sci USA 95:3100–3105

Arachidonate 5-lipoxygenase (P-5LO)

Phenotype

Develop normally and are healthy. No difference in their reaction to endotoxin shock, however resist the lethal effects of shock induced by platelet-activating factor. Inflammation induced by arachidonic acid is markedly reduced.

References

Chen XS, Sheller JR, Johnson EN, Funk CD (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372:179–182

Thrombopoietin

Phenotype

TPO –/– and c-mpl –/–: both exhibit a 90% reduction in megakaryocyte and platelet levels; but even with these small platelet levels the mice do not have excessive bleeding; all platelets which are present are morphologically normal + functionally; *in vivo* TPO is required for control of megakaryocyte and platelet number but not for their maturation.

References

Lawler J, Sunday M, Thibert V, Duquette M, George EL (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992

Thrombospondin-1

Phenotype

Normal thrombin-induced platelet aggregation; increase in circulating number of white blood cells; TSP-1 is involved in normal lung homeostasis.

References

Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, Hynes RO (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992

Chapter C
Activity on urinary tract[1]

[1] Reviewed by M. Hropot, contributions to the first edition by R. Greger, further contributions by H. Gögelein, M. Hropot and M. Bleich.

C.1
Diuretic and saluretic activity

C.1.1
In vitro methods

C.1.1.1
Carbonic anhydrase inhibition *in vitro*

PURPOSE AND RATIONALE

Acetazolamide (Diamox®) was one of the first synthetic non-mercurial diuretics. The mode of action was found to be inhibition of carbonic anhydrase. Carbonic anhydrase is a zinc-containing enzyme that catalyzes the reversible hydration (or hydroxylation) of CO_2 to form H_2CO_3 which dissociates non-enzymatically into HCO_3^- and H^+. At least three isoenzymes, designated as I, II and II or A, B and C, are known to exist. The chemistry, physiology and pharmacology of carbonic anhydrase has been extensively reviewed by Maren (1967). In spite of the fact that newer diuretics are based on

other modes of action, the test for inhibition of carbonic anhydrase should be performed for evaluation of a new compound. Moreover, the specific use of carbonic anhydrase inhibitors as antiglaucoma drugs has been described (Friedland and Maren 1984; Caprioli 1985). The mechanism by which carbonic anhydrase inhibitors lower intraocular pressure is through a reduction in aqueous humor formation, by affecting electrolyte and water balance in the nonpigmented ciliary epithelium (Friedland and Maren 1984; Caprioli 1985). Although many methods to measure carbonic anhydrase activity have been developed (Philpot and Philpot 1936), the micro method described by Maren (1960) is relatively simple, sensitive and reliable. The enzyme source are red cells, a rich source of the same isoenzymes found in the eye (Maren 1967; Armstrong et al. 1966; Wistrand et al. 1986; Wistrand and Knuutila 1980).

PROCEDURE

Materials and solutions

- phenol red indicator solution:
  12.5 mg phenol red/liter 2.6 mM $NaHCO_3$, pH 8.3 + 218 mM Na_2CO_3
- 1 M sodium carbonate/bicarbonate buffer, pH 9.8
- Enzyme: Carbonic anhydrase from dog blood; Blood is collected into a heparinized tube and diluted 1:100 with deionized water.
- Equipment
  - Reaction vessel – custom made by Labglass Inc., Vineland, NJ, USA
  - Monostat bench mounted flowmeter
  - 30% CO_2 – M&G Gases, Branchburg, NJ, USA

Assay

CO_2 flow rate is adjusted to 30 (45) ml/min. The following solutions are added to the reaction vessel:

400 µl phenol red indicator solution
100 µl enzyme
200 µl H_2O or appropriate drug concentration after 3 min for equilibration:
100 µl carbonate/bicarbonate buffer is added.

The following parameters are determined in duplicate samples:

T_u = (uncatalyzed time) = time for the color change to occur in the absence of enzyme.
T_e = (catalyzed time) = time for the color change to occur in the presence of the enzyme.
$T_u - T_e$ = enzyme rate
T_i = enzyme rate in the presence of various concentrations of inhibitor

EVALUATION

Percent inhibition of carbonic anhydrase is calculated according to the following formula:

$$\% \text{ Inhibition} = 1 - \frac{(T_u - T_e) - (T_i - T_e)}{T_u - T_e} \times 100$$

Standard data:

Compound	IC_{50} [M]
Acetazolamide	9.0×10^{-9}
Chlorothiazide	9.0×10^{-7}

CRITICAL ASSESSMENT OF THE METHOD

Determination of carbonic anhydrase inhibition is of value to characterize the activity spectrum of sulfonamide diuretics.

MODIFICATIONS OF THE METHOD

Landolfi et al. (1997) reported a modified procedure for the measurement of carbonic anhydrase activity. The measure of carbonic anhydrase activity is based on the rate of CO_2 hydration by the enzyme. Such transformation was monitored by a procedure which consists in the measure of time necessary for the pH of an appropriate buffer to decrease from 8 to 7.5 in the presence of a constant CO_2 flow: such time period is dose-dependently reduced by the addition of the enzyme and further modified in the presence of carbonic anhydrase inhibitory compounds.

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C.1.1.2 Patch clamp technique in kidney cells[2]

PURPOSE AND RATIONALE

In the different parts of the kidney (proximal tubules, distal tubules, collecting ducts) fluid is reabsorbed and substances may be transported either from the tubule lumen to the blood side (reabsorption) or vice versa (secretion). Besides active transport and coupled transport systems, ion channels play an important role in the function of kidney cells. The various modes of the patch clamp technique (cell-attached, cell-excised, whole-cell mode) (Neher and Sakmann 1976; Hamill et al. 1981) allow the investigation of ion channels. In addition, the investigation of other electrogenic transport mechanisms, such as the sodium-coupled alanine transport can be studied.

PROCEDURE

The patch clamp technique can be applied to cultured kidney cells (Merot et al. 1988), to freshly isolated kidney cells (Hoyer and Gögelein 1991) or to cells of isolated perfused kidney tubules (Gögelein and Greger 1984). The latter method shall be described in more detail.

Segments of late superficial proximal tubules of rabbit kidney are dissected and perfused from one end with a perfusion system (Burg et al. 1966; Greger and Hampel 1981). The non-cannulated end of the tubule is freely accessible to a patch pipette. Under optical control (differential interference contrast optics with 400× magnification) the patch pipette can be moved through the open end into the tubule lumen and is brought in contact with the brush border membrane. After slight suction of the patch electrode, gigaseals form instantaneously and single potassium or sodium channels can be recorded in the cell-attached or inside-out cell-excised mode (Gögelein and Greger 1984; Gögelein and Greger 1986a).

In order to obtain exposed lateral cell membranes suitable to the application of the patch clamp method, pieces of the tubule are torn off by means of a glass pipette (diameter about 40 µm). As to facilitate the tearing off, the tubules are incubated for about 5 min in 0.5 g/l collagenase (Sigma, C 2139) at room temperature. After tearing off part of the cannulated tubule, clean lateral cell membranes are exposed at the non-cannulated end. The patch pipette can be moved to the lateral cell membrane and gigaseals can be obtained. It was possible, to investigate potassium channels (Gögelein and Greger 1987) and nonselective cation channels (Gögelein and Greger 1986b) in these membranes.

As cells are still part of an epithelial layer and, therefore, are intracellularly coupled, the whole-cell technique is not appropriate in this preparation. On the other hand, cotransport systems can only be investigated by the whole-cell method because the transport rate of a single event is much too small to be resolved in a similar manner as single ion channel events. Consequently, cells of rabbit proximal tubules are isolated as described in detail elsewhere (Hoyer and Gögelein 1991; Heidrich and Dew 1977). After cervical dislocation the kidneys are rapidly excised and placed in ice-cold solution [mmol/l]: 150 K-cyclamate, 10 HEPES, 1 $CaCl_2$, 1 $MgCl_2$, pH 7.4. The following steps are performed on ice: After decapsulation, superficial cortical slices of about 0.5 mm thickness are dissected and minced with a scalpel. The tissue is homogenized in a Dounce homogenizer by three strokes with a loose-fitting pestle. The homogenate is then poured through graded sieves (250, 75 and 40 µm) to obtain a population of single cells. Since the predominant tubule section of the cortex of the rabbit kidney is the pars convoluta of the proximal tubule, it can be concluded that the majority of the isolated cells in the cell suspension are of proximal tubule origin. By light microscopy cells are identified by long microvilli distributed over the entire cell surface and can easily be discriminated from remaining erythrocytes, cell detritus and tubular fragments.

By application of the whole-cell mode of the patch clamp technique to freshly isolated cells of convoluted proximal tubules, the sodium-alanine cotransport system could be investigated in detail (Hoyer and Gögelein 1991).

EVALUATION

In isolated perfused renal tubules, concentration response curves of drugs which inhibit ion channels can be obtained with the patch clamp technique. In isolated cells of the proximal tubule, the whole-cell mode of the patch clamp technique enables the investigation of the sodium-alanine cotransport system. The apparent K_m values for sodium and L-alanine can be recorded.

MODIFICATIONS OF THE METHOD

Schlatter (1993) recorded membrane voltages of macula densa cells with the fast or slow whole-cell patch-clamp method. The effects of diuretics and the conductance properties of these cells were examined.

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C.1.1.3 Perfusion of isolated kidney tubules[3]

PURPOSE AND RATIONALE

The various tubule segments: proximal tubule (PT, S1–S3); descending thin limb of the loop of Henle (DTL); ascending thin limb of the loop of Henle (ATL); thick ascending limb of the loop of Henle (TAL); distal convoluted tubule (DCT); connecting tubule (CNT); cortical collecting duct (CCD); medullary collecting duct (MCD); papillary collecting duct (PCD) have different functional properties. The *in vitro* perfusion of isolated tubule segments (Burg et al. 1966) is the method of choice if one has to identify the site and the mechanism of action of a pharmacological agent which has been shown to act on kidney function in clearance and micropuncture studies.

PROCEDURE

After its invention by Burg et al. (1966) this technique has been used successfully in the kidney tubule segments of several species: man; rabbit; rat; mouse; hamster; snake; birds etc. The tubule segments are dissected from thin kidney slices (<1 mm thickness). Usually dissection can be done using sharpened forceps or needles without the addition of proteases (collagenase). The segment is identified by its anatomical location and by its appearance. A 20–50× lens is used for dissection. Dark field illumination is helpful for the identification of the segment under study. PT, TAL, DCT, CNT, CCD, MCD can all be dissected quite easily. The dissection of DTL, ATL and PCD is much more difficult because these segments are damaged easily by the mechanical dissection. Dissection is usually performed at 4 °C in a Ringer type solution.

The dissected segment is transferred into the perfusion chamber by a transfer pipette. The perfusion chamber is mounted in the stage of an inverted microscope (20–400×). The chamber is usually kept at 37 °C, and the bath perfusate is also preheated to this temperature. The bath perfusate will depend on the tubule segment under study. In most instances it will contain HCO_3^- and will be bubbled with CO_2. The metabolic substrate will be acetate for PT and D-glucose for TAL, CCD etc. The actual perfusion is performed with two sets of concentric glass pipettes, one set at the perfusion end; and one at the collection end of the segment. These pipettes are manufactured with special glass forges. The most refined one has been designed by Hampel and Greger. The glass tube is rotated at approximately 1 rps, is moved in perpendicular direction by a remote control, and the heating filament is moved in xy direction also by a remote control. The shaping of the glass is observed continuously by a lens (5–50×). The pulling force is provided by weights fixed on the lower end of the glass tube. The pipettes are cut either by a diamond or by the pulling force of a small glass bead, fixed on the edge of a vertical platinum filament and melted sidewise on the pipette. When the heating current of the platinum filament is switched off the filament retracts and brakes the pipette a the desired site. Greger and Hampel (1981) have modified the original perfusion system of Burg and co-workers. Their device is optimized inasmuch as it guarantees concentric alignment of the various pipettes. The forward and backward movement is controlled by small electric motors. At the perfusion side they use 4 concentric pipettes. The outermost one contains sylgard and is driven over the perfused end of the tubule in order to seal this end. The tubule is held by a holding pipette with appropriate dimensions. The tubule is sucked into this pipette up to the constriction. Then the perfusion pipette with a tip diameter smaller than the inner diameter of the perfused segment is advanced into the segment held by the holding pipette. The perfusion pipette is put under hydrostatic pressure of a few to

[3] Contribution by R. Greger (first edition) and M. Bleich (second edition).

100 cm to achieve a perfusion rate of 1–20 nl/min. Usually the collapsed tubule lumen opens when the perfusion pipette is advanced. The pipette is advanced in the lumen until it reaches an area of the segment where it appears intact by inspection (200–400×). Within its lumen the perfusion pipette contains yet another pipette, the fluid exchange pipette. With this pipette the composition of the perfusate can be replaced very rapidly (Greger and Schlatter 1983). The collection end of the tubule segment is sucked into a holding pipette. A sylgard pipette is advanced to seal the collection site. The holding pipette at the collection site will contain mineral oil in flux measurements. Then a collection pipette is advanced through the oil to quantitatively collect the perfusate delivered by the tubule.

The **measured parameters** can be as follows:

Flux measurements (Schafer et al. 1974). The collection rate (V_c, nl/min) can be measured by the constant bore collection pipette by timed collections. Radioactive tracers can be added to the lumen or bath fluid. For instance, radioactively labelled inulin can be added to the perfusate (In_p) and can be used to measure volume absorption (ΔV = perfusion rate ($V_i - V_c$). Unidirectional fluxes, bath to lumen and lumen to bath, for any given substance can be quantified, and permeabilities (P_x) can be determined:

$$P_x = (V_i - V_c)\, L^{-1} [\ln (x_p In_c x_c^{-1} In_p) + 1]$$

where L is the length of the segment; x_p and x_c are the concentrations of x in the perfusate and in the collected fluid; and In_c is the inulin concentration in the collected fluid. Net fluxes of x can be determined as the difference of the unidirectional fluxes or by the chemical determination of Δx (perfusate – collected fluid). This requires very sensitive methods. Electron probe analysis of Na^+, K^+, Ca^{2+}, Mg^{2+} etc. has been used to determine the net transport of these ions in various tubule segments (Wittner et al. 1988). Flux studies are usually performed at low luminal perfusion rates of a few nl/min. Substances under study can be added to the luminal and bath perfusate, and paired data can be obtained under control and experimental conditions (Burg and Green 1973; Stoner et al. 1974; Burg 1980; Burg and Stoner 1976; Dillingham et al. 1993).

Transepithelial electrical measurements. The perfusion pipette can be connected to the high impedance input of an electrometer. The voltage is referenced to the grounded bath. The connections are usually made with agar bridges (80 g agar in 1 l Ringer's solution), and appropriate corrections for liquid junction voltages must be applied. With identical solutions in the bath and in the lumen and with high luminal perfusion rates (> 10 nl/min), any transepithelial voltage (V_{te}) must be caused by active transport = active transport potential (Frömter 1984). Hence, the effectiveness of putative inhibitors of active transport can also be examined by the measurement of V_{te}. According to Ohm's law the determination of the flux of ions also requires the measurement of transepithelial resistance. Greger (1981) has introduced a method which utilizes a dual channel perfusion pipette, made of Q-shaped glass. One channel is used for perfusion and the other for current (I_{te}) injection. The current is defined by a resistor chosen such that the deflection in V_{te} generated by this pulse is in the order of 10–20 mV. Transepithelial resistance (R_{te}) can now be calculated from ΔV_{te} and I_{te}. The ratio of V_{te} and R_{te} is called equivalent short circuit current. It is directly proportional to active transport (Greger 1985). The measurement of V_{te} and R_{te} is much more efficient than flux studies for pharmacological screening, provided that the process under study produces a transepithelial voltage. Several substances can be examined in one single tubule in strictly paired fashion (Schlatter et al. 1983; Wangemann et al. 1986). The time resolution of the measurements is on the order of 1 s, whereas that of flux studies is several minutes at best.

Intracellular electrical measurements. Greger and Schlatter (1983) have developed a method for the use of impalement techniques in the isolated perfused tubule. Very fine tip microelectrodes (Ø < 100 nm) are used to impale the tubule cell across the basolateral membrane. The actual impalement is performed by a piezo stepper which accelerates the microelectrode to high speed, which makes it possible to penetrate the rigid basal membrane. The simultaneous measurement of V_{te}, R_{te}, and basolateral membrane voltage (V_{bl}) allows for a complete analysis of voltages and resistances (Greger 1985; Ullrich and Greger 1985). Ion selective microelectrodes can also be used in impalement studies, and the cytosolic ion activities for e.g. Na^+, K^+, Cl^- can also be determined (Greger 1985). These methods are all rather difficult to perform. They are of high relevance for the understanding of the function of a given tubule segment and for the detailed description of the mechanism of action of a drug, which, in preceding studies has been shown to act in a given tubule segment.

Patch clamp studies. The combination of *in vitro* perfusion of renal tubules and patch clamp analysis of ion channels in the luminal and basolateral membranes is described in Sect. C.1.1.2.

Fluorescent dyes in the isolated perfused tubule. Several fluorescent dyes for the monitoring of Na^+, K^+,

Cl^-, Ca^{2+}, pH have become available during the past few years. These dyes can be used in the *in vitro* perfused tubule (Nitschke et al. 1991). The inverted microscope is equipped with an appropriate illumination and filter wheel for excitation. The emission is measured by photon counting or by a video camera. When compared with impalement methods, these techniques are probably easier for routine use.

EVALUATION

For each of the above protocols paired measurements of one or several given parameters of tubule transport are obtained under control conditions and in the presence of a substance under study. Also concentration response curves can be obtained in one single preparation (Schlatter et al. 1983; Wangemann et al. 1986; Wittner et al. 1987). Intracellular measurements are usually required to define the mechanism of action (Greger 1985). Especially the electrical and optical measurements have a very high reproducibility. For screening usually 3 preparations are sufficient. Approximately 10 preparations are required for concentration response curves.

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C.1.1.4 Isolated perfused kidney

PURPOSE AND RATIONALE

Isolated kidney is a good tool for studying proximal tubule, but of limited value for distal tubule function. The kidney can be perfused *in situ* and isolated *in vitro*. The isolated kidney can be perfused by a pump using blood or plasma-like solutions. One specific problem of the blood-perfused dog kidney *in vitro* is its instability. After only 1 h of perfusion, glomerular filtration and renal blood flow decline markedly. It was reported that *in situ*-perfused isolated dog kidney seems to be more stable. In isolated perfused rat kidney plasma-like solutions are used for perfusion. This system, by inclusion of a dialyzing unit, provides optimal conditions for maintaining a constant electrolyte composition of the perfusate. However, function of distal tubule is also grossly impaired in this rat model. The isolated kidney does not acidify tubular fluid, and the concentrating ability is reduced.

PROCEDURE

Kidneys are obtained from anaesthetized male rats with a body weight of 300 to 400 g. The donor animals are fasted overnight prior to surgery, but have free access to water. After the abdominal cavity is exposed by a

ventricular incision, the right ureter is cannulated with PE-50 polyethylene tubing and heparin is injected into the vena cava (500 U/kg body weight). The venous cannula is introduced into the vena cava below the right renal vein. The right kidney is freed from the perirenal fat, not disrupting the renal capsule. The renal artery is cannulated via the superior mesenteric artery without interruption of flow. Thereafter, the kidney is continuously perfused with a perfusion solution fed from the gravity system situated 130 cm above the cannula. Ligatures around the renal artery and vena cava above the renal pedicle are tied. The kidney is then removed from the animal and placed in a Plexiglas chamber. A perfusion pressure of 80–90 mm Hg in the renal artery is maintained by adjusting the speed of the perfusion pump. For more details see references.

EVALUATION

After the equilibration period, clearance periods of 20 min are used. Urine samples are collected and perfusate is obtained at midpoint of the clearance period for the evaluation of overall kidney function. For determination of glomerular filtration rate (GFR) and fluid transport, ^{3}H-labelled polyethylene glycol is added to a modified Krebs-Henseleit bicarbonate buffer. Electrolytes are determined in urine by standard flame photometry. Fractional excretions of water, electrolytes and test compounds are calculated.

MODIFICATIONS OF THE METHOD

Tarako et al. (1991) evaluated oxygen supply and energy state in the isolated perfused rat kidney.

Metabolic activities of the isolated perfused rat kidney were described by Nishiitsutsuji-Uwo et al. (1967).

Cox et al. (1990) used the isolated perfused rat kidney as a tool in the investigation of renal handling and effects of nonsteroidal anti-inflammatory drugs.

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C.1.2 *In vivo* methods

C.1.2.1 Diuretic activity in rats (LIPSCHITZ test)

PURPOSE AND RATIONALE

A method for testing diuretic activity in rats has been described by Lipschitz et al. (1943). The test is based on water and sodium excretion in test animals and compared to rats treated with a high dose of urea. The "Lipschitz-value" is the quotient between excretion by test animals and excretion by the urea control.

PROCEDURE

Male Wistar rats weighing 100–200 g are used. Three animals per group are placed in metabolic cages provided with a wire mesh bottom and a funnel to collect the urine. Stainless-steel sieves are placed in the funnel to retain feces and to allow the urine to pass. The rats are fed with standard diet (Altromin® pellets) and water ad libitum. Fifteen hours prior to the experiment food and water are withdrawn. Three animals are placed in one metabolic cage. For screening procedures two groups of three animals are used for one dose of the test compound. The test compound is applied orally at a dose of 50 mg/kg in 5.0 ml water/kg body weight. Two groups of 3 animals receive orally 1 g/kg urea. Additionally, 5 ml of 0.9% NaCl solution per 100 g body weight are given by gavage. Urine excretion is recorded after 5 and after 24 h. The sodium content of the urine is determined by flame photometry. Active compounds are tested again with lower doses.

EVALUATION

Urine volume excreted per 100 g body weight is calculated for each group. Results are expressed as the "Lipschitz-value", i.e., the ratio T/U, in which T is the response of the test compound, and U, that of urea treatment. Indices of 1.0 and more are regarded as a positive effect. With potent diuretics, Lipschitz values of 2.0 and more can be found. Calculating this index for the 24 h excretion period as well as for 5 h indicates the duration of the diuretic effect. Similar to urine volume, quotients can be calculated for sodium excretion.

Dose-response curves can be established using various doses. Loop diuretics are characterized by a steep dose-response curve. Saluretic drugs, like hydrochlorothiazide, show Lipschitz values around 1.8, whereas loop diuretics (or high ceiling diuretics) like furosemide, bumetanide or piretanide reach values of 4.0 and more.

CRITICAL ASSESSMENT OF THE METHOD

The Lipschitz test has been proven to be a standard method and a very useful tool for screening of potential diuretics.

MODIFICATIONS OF THE METHOD

The method has been modified in various ways by several authors. Cummings et al. (1960) recommended a sequential procedure with criteria for acceptance or rejection of test drugs. Kau et al. (1984) recommended a method for screening diuretic agents in the rat using normal saline (4% body weight) as hydrating fluid.

Homozygous Brattleboro rats exhibit symptoms of diabetes insipidus (Valtin et al. 1965). The condition is due to the failure of hypothalamic neurons to produce vasopressin, which is due to a single base point deletion in the vasopressin gene (Schmale and Richter 1984). The abnormal quinine drinking aversion in the Brattleboro rat with diabetes insipidus is reversed by a vasopressin agonist (Laycock et al. 1994).

These animals can be used to study vasopressin agonism and antagonism and the aquaretic effects of synthetic drugs.

Klatt et al. (1975) described a method of collecting urine excreted by cats. On the basis of urine funnel used in rats, an appropriate larger metabolism cage made out of transparent, rigid polyvinyl chloride was used. The cage was improved by a built-in sieve cone which assured good separation of urine and feces. A device to measure and record the time and amount of voided urine was attached. Urine was collected in a vessel with a hose connection from the bottom to a pressure sensor. An attached overflow tube could be occluded. The initial pressure of the sensor was fed into a linear recorder. Before the test, the recorder was calibrated with a sufficient amount of distilled water to adjust the number of division intervals for direct measurement of voided urine in milliliters. This allowed calculation of the time point of voiding from the chart speed.

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C.1.2.2 Saluretic activity in rats

PURPOSE AND RATIONALE

Excretion of electrolytes is as important as the excretion of water for treatment of peripheral edema and ascites in congestive heart failure as well as for treatment of hypertension. Potassium loss has to be avoided. As a consequence, saluretic drugs and potassium-sparing diuretics were developed. The diuresis test in rats was modified in such a way that potassium and chloride as well as osmolality are determined in addition to water and sodium. Ratios between electrolytes can be calculated indicating carbonic anhydrase inhibition or a potassium sparing effect.

PROCEDURE

Male Wistar rats weighing 100–200 g fed with standard diet (Altromin® pellets) and water ad libitum are used. Fifteen hours prior to the test, food but not water is withdrawn. Test compounds are applied in a dose of

50 mg/kg orally in 0.5 ml/100 g body weight starch suspension. Three animals are placed in one metabolic cage provided with a wire mesh bottom and a funnel to collect the urine. Two groups of 3 animals are used for each dose of a test drug. Urine excretion is registered every hour up to 5 h. The 5-h urine is analyzed by flame photometry for sodium and potassium and argentometrically by potentiometrical end point titration (Chloride-Titrator Aminco) for chloride. To evaluate compounds with prolonged effects the 24 h urine is collected and analyzed. Furosemide (25 mg/kg p.o.), hydrochlorothiazide (25 mg/kg p.o.), triamterene (50 mg/kg p.o.), or amiloride (50 mg/kg p.o.) are used as standards.

EVALUATION

- The sum of Na^+ and Cl^- excretion is calculated as parameter for saluretic activity.
- The ratio Na^+/K^+ is calculated for natriuretic activity. Values greater than 2.0 indicate a favorable natriuretic effect. Ratios greater than 10.0 indicate a potassium-sparing effect.
- The ratio

$$\frac{Cl^-}{Na^+ + K^+}$$

(ion quotient) is calculated to estimate carbonic anhydrase inhibition.
- Carbonic anhydrase inhibition can be excluded at ratios between 1.0 and 0.8. With decreasing ratios slight to strong carbonic anhydrase inhibition can be assumed.

MODIFICATIONS OF THE METHOD

Adrenalectomized rats treated with DOCA or aldosterone can be utilized to test **aldosterone antagonists**. Spironolactone has no effect in the absence of a mineralocorticoid, but reverses in a dose-related manner the effect of DOCA on the Na^+/K^+ ratio in the urine (Kagawa et al. 1957; Bicking et al. 1965).

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C.1.2.3 Diuretic and saluretic activity in dogs

PURPOSE AND RATIONALE

Dogs have been extensively used to study renal physiology and the action of diuretics. Renal physiology of the dog is claimed to be closer to man than that of rats. Oral absorbability of diuretic substances can appropriately be studied in dogs. Using catheters, interval collections of urine can be made with more reliability than in rats. Simultaneously, blood samples can be withdrawn to study pharmacokinetics.

PROCEDURE

Beagle dogs of either sex have to undergo intensive training to be accustomed to accept gavage feeding and hourly catheterization without any resistance. The dogs are placed in metabolic cages. At least 4 dogs are used as controls receiving water only, as standard controls (1 g/kg urea p.o. or 5 mg/kg furosemide p.o.) or the test drug group. Twenty-four hours prior to the experiment food but not water is withheld. On the morning of the experiment, the urine bladder is emptied with a plastic catheter. The dogs receive 20 ml/kg body weight water by gavage, followed by hourly doses of 4 ml/kg body weight drinking water. The bladder is catheterized twice in an interval of 1 h and the urine collected for analysis of initial values. Then, the test compound or the standard is applied either orally or intravenously. Hourly catheterization is repeated over the next 6 h. Without further water dosage the animals are placed in metabolic cages overnight. Twenty-four hours after dosage of the test compound, the dogs are catheterized once more and this urine together with the urine collected over night in the metabolic cage registered. All urine samples are analyzed by flame photometry for sodium and potassium and by argentometry (Chloride Titrator Aminco) for chloride content. Furthermore, osmolality is measured with an Osmometer.

EVALUATION

Urine volume, electrolyte concentrations and osmolality are averaged for each group. The values are plotted against time to allow comparison with pretreatment values as well as with water controls and standards. The non-parametric U-test is used for statistical analysis.

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C.1.2.4 Clearance methods[4]

PURPOSE AND RATIONALE

Investigations of clearance represent indirect methods for the evaluation of renal function and provide information on the site of action of diuretics and other pharmacological agents within the nephron. The discovery of the countercurrent multiplier system as the mechanism responsible for the concentration and dilution of the urine has been the prerequisite for the identification of the site of action of diuretic drugs. A drug that acts solely in the proximal convoluted tubule, by causing the delivery of the increased amounts of filtrate to the loop of Henle and the distal convolution, would augment the clearance of solute-free water (C_{H_2O}) during water diuresis and the reabsorption of solute-free water (T_{CH_2O}) during water restriction. In contrast, drugs that inhibit sodium reabsorption in Henle's loop would impair both C_{H_2O} and T_{CH_2O}. On the other hand, drugs that act only in the distal tubule would reduce C_{H_2O} but not T_{CH_2O}.

PROCEDURE

Clearance experiments are performed either in conscious or anaesthetized beagle dogs under conditions of water diuresis and hydropenia. The status of water diuresis and hydropenia may be accomplished as described by Suki et al. (1965). Water diuresis is induced by oral administration of 50 ml of water per kg body weight and maintained by continuos infusion into jugular vein of 2.5% glucose solution and 0.58% NaCl solution at 0.5 ml/min per kg body weight. When water diuresis is well established, the glucose infusion is discontinued and control urine samples are collected by urethral catheter. Blood samples are obtained in the middle of each clearance period. After the control period, compounds to be tested are administered and further clearance tests are performed.

Hydropenia is induced by withdrawing the drinking water 48 h before experiment. On the day before the experiment 0.5 U/kg body weight of vasopressin in oil is injected intramuscularly. On the day of the experiment 20 mU/kg vasopressin is injected i.v., followed by infusion of 50 mU/kg per hour vasopressin. To accomplish constant urine flow 5% NaCl solution is infused at 1 ml/min per kg body weight up to i.v. administration of a compound to be tested, followed by i.v. infusion of 0.9% NaCl solution at a rate equal to the urine flow. Glomerular filtration rate (GFR) and renal plasma flow (RPF) are measured by the clearance of inulin and para-aminohippurate, respectively. Therefore, appropriate infusion of inulin (bolus of 0.08 g/kg followed by infusion of 1.5 mg/kg per min) and para-aminohippurate (bolus 0.04 g/kg followed by infusion of 0.3 mg/kg per min) are initiated. Inulin and para-aminohippurate are measured according to Walser et al. (1955) and Smith and al (1945), respectively.

EVALUATION

The following parameters may be determined: water and electrolyte excretion, GFR, RPF, C_{H2O}, T_{CH2O} and plasma renin activity. Results of test compound are compared statistically with control and standard drug treated animals.

MODIFICATIONS OF THE METHOD

Rönnhedh et al. (1996) described a simple method to perform serial renal clearance studies without urine collection in rats. This was applied to non-radiolabeled para-aminohippurate sodium and iothalamate sodium which were used respectively to estimate renal blood flow and glomerular filtration rate.

Gabel et al. (1996) described fast and accurate assays for measuring glomerular filtration rate and effective renal blood flow in conscious rats. An enzymatic method was developed for the determination of inulin and a colorimetric method was developed for determination of p-aminohippurate in the plasma and urine of rats.

Hropot et al. (1985) described clearance methods in monkeys. Chimpanzees weighing 30.7 ±10.6 kg were anesthetized with 1 mg/kg Sernylan i.m. Food was withdrawn 24 h prior to the experiment and the animals received only tap water ad libitum. In the morning before the experiment, the urinary bladder of the animals was emptied by catheterization. The urine was discarded. To determine the glomerular filtration rate (inulin clearance), a bolus injection of 50 mg/kg inulin i.v. was given and followed by a continuous infusion of 3 ml/min inulin dissolved in Ringer lactate solution. After an equilibrium of 60 min, urine and blood samples were collected for two control clear-

[4] Contribution by M. Hropot.

ance periods of 30 min each. The control periods were followed by intravenous administration of the test preparation in a dose of 20 mg/kg. Thereafter, urine and blood samples were collected during 6 clearance periods. The following parameters were determined: urine excretion, inulin clearance and urate clearance [ml/kg/min], fractional excretion of urate and plasma urate [mmol/l].

Tanaka et al. (1990) evaluated uricosuric and diuretic properties of diuretic agents using clearance studies in urate-loaded dogs and urate-loaded rabbits.

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C.1.2.5 Micropuncture techniques in the rat[5]

PURPOSE AND RATIONALE

Micropuncture techniques have been applied to the direct investigation of the effect of diuretics on single nephron function. The observed changes in tubular fluid reabsorptive rates and electrolyte concentrations can be used to asses the mechanism of action. The rat is the model of choice since proximal and distal tubules as well as collecting ducts are accessible for micropuncture.

[5] Contribution by M. Hropot.

PROCEDURE

Clearance and free-flow micropuncture studies are performed in rats with a body weight of about 250 g, anaesthetized by the intraperitoneal injection of thiopentone (Trapanal® 50 mg/kg). The animals are fasted for 16 h before the beginning of the experiment, but have free access to tap water. After anesthesia the animals are placed on a thermostatically heated table. Thereafter rats are tracheotomized and carotid artery and jugular vein are cannulated for blood pressure recording, blood sampling, and for infusion of compounds, respectively. The left kidney is carefully exposed by a flank incision, embedded in a small plastic vessel with cotton wool, and bathed with paraffin oil at 37 °C. The ureter is cannulated and rectal temperature monitored continuously. A bolus injection of 75 µCi inulin ^{3}H in 0.7 ml NaCl solution is given, followed by 0.85% NaCl solution at a rate of 2.5 ml/min per 100 g body weight. The sustained infusion delivers 75 µCi inulin ^{3}H per hour. The control puncture of tubules is performed 45 min after beginning of the intravenous infusion. The direct collection of tubular fluid samples from proximal and distal tubules is carried out with glass capillaries of 8 to10 µm external diameter using a micromanipulator and microscopic observation. Distal tubules are identified by intravenous injection of lissamine green. The control period is followed by the test period. After an equilibration period of 30 min with the compound to be tested, micropuncture is performed again and tubular fluid is collected. The uretral urine is collected and blood sampling is performed in the middle of each clearance period.

EVALUATION

The following parameters may be determined: inulin clearance (GFR), single nephron GFR, fractional delivery of water, sodium and potassium in proximal and distal tubules and in urine. All data are expressed as mean values ±SEM. Comparison of the effects of compounds to be tested with controls is performed by one way analysis of variance and by Student's *t*-test for paired and unpaired data.

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C.1.2.6 Stop-flow technique[6]

PURPOSE AND RATIONALE

This procedure is of considerable value in the localization of transport processes along the length of the nephron. During clamping of the ureter, glomerular filtration is grossly reduced. The contact time for the tubular fluid in the respective nephron segments increases, and the concentration of the constituents of tubular fluid should approximate the static-head situation. After release of the clamp, the rapid passage of the tubular fluid should modify the composition of the fluid only slightly. The first samples should correspond to the distal nephron segment, the latest to glomerular fluid. However, with introduction of the micropuncture technique, the stop-flow method appears less attractive.

PROCEDURE

This method can be performed in different animals during anesthesia and was originally described by Malvin et al. (1958). The ureter of an animal undergoing intense osmotic diuresis is clamped for several minutes allowing a relatively static column of urine to remain in contact with the various tubular segments for longer than the usual periods of time. Thus, the operation of each segment on the tubular fluid is exaggerated. Then the clamp is released, and the urine is sampled sequentially. Small serial samples are collected rapidly, the earliest sample representing fluid which had been in contact with the most distal nephron segment. Substances examined are administered along with inulin before the application of uretral occlusion. However, tubular segments downstream from the proximal segments may modify the tubular fluid during its egress.

EVALUATION

In each sample the concentration of a glomerular marker, such as inulin, and the concentration of the substance under study are measured. Fractional excretion of the substance and the glomerular marker are plotted versus the cumulative urinary volume.

MODIFICATIONS OF THE METHOD

Shinosaki and Yonetani (1989), Shinosaki et al. (1994) performed stop-flow studies on tubular transport of uric acid in rats treated with pyrazinoic acid, an inhibitor of tubular urate secretion.

Tanaka et al. (1990) used stop-flow experiments to test uricosuric and diuretic activities of new compounds in dogs.

[6] Contribution by M. Hropot.

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C.2 Impaired renal function

C.2.1 Chronic renal failure in the rat

PURPOSE AND RATIONALE

Chronic renal failure is a frequent pathological condition in man. An animal model as described by Acott et al. (1987) is of value to test new diuretics under these conditions.

PROCEDURE

Sprague-Dawley rats weighing 150–200 are anesthetized by i.m. injection of ketamine (40 mg/kg) and droperidol/fentanyl (Inovar) 0.25 mg/kg. Through a 6-cm midline incision in the abdominal wall the small bowel and cecum are lifted and placed on saline-soaked gauze sponges. The exposed right kidney is dissected from the retroperitoneal area and the vascular and ureteric pedicles are ligated with 2–0 silk sutures, transected, and the kidney is removed. The renal artery of the left kidney is dissected into the hilum to expose the three main segmental renal arteries. The kidney is not dissected out of the peritoneum. The anterior caudal branch of the artery is then temporarily ligated to establish the volume of renal tissue supplied. The area of ischemia becomes demarcated within 10–15 s. If this approximates 1/4 to 1/3 of the kidney, a permanent ligature is placed. The viscera are then carefully replaced in the abdomen and peritoneum and linea alba are closed with a continuous suture. The skin is closed with stainless-steel clips.

Blood for serum creatinine is collected by retro-orbital puncture under anesthesia at various time intervals up to 12 months. In association with this, 24-hour urines are collected for measurement of creatinine, protein, and specific gravity.

EVALUATION

Serum creatinine increases up to 500 µM/l after 12 months, whereas creatinine clearance decreases. Significantly increased urine volumes are accompanied by decreased urine specific gravity indicating a decreased concentrating ability. Proteinuria is significantly increased. Terminal uremia occurs after 14–15 months.

CRITICAL ASSESSMENT OF THE METHOD

The method may be used for special pharmacological studies as well as for evaluation of renal toxicity of new chemicals.

MODIFICATIONS OF THE METHOD

Sancho et al. (1989) used a similar procedure in rats ligating two of the three terminal branches of the left renal artery, followed by right nephrectomy.

Freeman (1971) induced azotemia combined with hypothermia in rats by ligation of the urinary bladder at the base.

William et al. (1997) described renal ischemia-reperfusion injury in rats. The animals were anesthetized and subjected to 45 min of bilateral renal occlusion using atraumatic vascular clamps before renal perfusion was reestablished. After various time interval (up to 1 week) blood urea nitrogen, creatinine and myeloperoxidase activity in the kidney were determined. The protective effects of an intracellular adhesion molecule monoclonal antibody were tested.

Ishidoya et al. (1995), Klahr and Morrissey (1997) induced interstitial renal fibrosis by unilateral ureteral obstruction in Sprague Dawley rats and tested the effect of ACE inhibitors and angiotensin II receptor antagonists.

Hartenbower and Coburn (1972) described a method for producing chronic renal insufficiency in the *chick*. By urethral ligation, the function of one kidney was completely eliminated, and the functional mass of the other was reduced by two-thirds. The method resulted in elevation of plasma concentration of uric acid, the major product of protein catabolism in avian plasma, to levels 2–4 times normal for periods as long as 3 weeks.

Two to 3 weeks old White Leghorn cockerels are anesthetized with ether, and the abdominal feathers are clipped. The chick is placed supine on a small operating board with hips flexed and legs extended over the head. An incision is made along the left side of the abdomen extending into the peritoneal cavity. Self-retaining retractors are used to maintain exposure. The right ureter is identified and ligated just proximal to its junction with the cloaca. The left ureter and renal vein are ligated with a single suture near the middle of the left kidney.

The degree of azotemia is assessed by measuring uric acid levels in blood samples 0.2–0.3 ml, obtained by cardiac puncture at intervals of 2–6 days after surgery.

Uric acid blood levels are compared between operated and sham-operated animals. Histological examination is performed after sacrifice of the animals.

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C.2.2 Chronic renal failure after subtotal (five-sixths) nephrectomy in rats

PURPOSE AND RATIONALE

Subtotal (five-sixths) nephrectomy in rats has been used by many authors as model for chronic renal failure. Levine et al. (1997) used this model to evaluate the hypothesis that under these conditions endogenous angiotensin II modulates *in vivo* bicarbonate reabsorption (J_{tCO_2}) in distal tubules via H^+-adenosinetriphosphatase and Na^+/H^+-exchange. Bicarbonate reabsorption (J_{tCO_2}) in distal tubules is significantly increased in five-sixths nephrectomized rats.

PROCEDURE

Surgical procedure

Male Sprague-Dawley rats weighing 230–280 g are anesthetized with halothane via a face mask. Prior to the first incision, the animal is given 20 ml/kg normal saline subcutaneously. On a heated table, a midline laparatomy is performed 2 cm below the xiphoid bone cartilage and the abdomen is opened. The right kidney is isolated and brought out of the abdomen by grasp-

ing the fat at the lower pole of the kidney. This fat is blunt dissected (sparing the adrenal gland), and three silk 4-0 ligatures are passed under the ureter, renal artery, and renal vein, tying one distally and two proximally. The ureter, renal artery and renal vein are then cut between the ligatures, the proximal ties are cut short, and the distal tie is pulled out, removing the kidney. After the area is checked for bleeding, the left kidney is the similarly isolated and brought out of the abdomen. The fat around the kidney is blunt dissected, avoiding excessive handling of the kidney or damage to the ureter, and the left and right renal poles are removed (two-thirds nephrectomy). The left remnant kidney is then returned to the abdomen and moistened with 5 ml of normal saline. The abdomen is closed with sutures and the skin with autoclips. Sham rats undergo the same procedure, except that the kidneys are only touched by the instruments. All rats are allowed 13–16 days recovery prior to microperfusion.

Microperfusion experiments

The rats are anesthetized with 100 mg/kg thiobutabarbital sodium and placed on a heated operating table. After tracheostomy, using a PE-240 tubing, the left carotid artery is cannulated for continuous blood pressure measurement and collection of blood for acid-base and electrolyte analyses while the left jugular vein is cannulated with three lines for infusion of fluid, pentobarbital sodium anesthetic and 10% Lissamine green (Levine et al. 1996). The left kidney is exposed by flank incision, carefully dissected from the adrenal gland, and immobilized in a stainless steel cup covered with mineral oil. The ureter is catheterized with PE-50 tubing to ensure proper urine flow.

To replace surgical fluid losses, the rats are infused at 1% body wt/h for 30 min via the jugular vein with donor plasma from control rats. The animals are then maintained on 0.9% saline at 1% body wt/h for the remainder of the experiment.

Two-loop perfusable surface distal tubules are identified by injecting a bolus of 1% Lissamine green into surface proximal loops and observing its passage through the nephron. The distal tubules are perfused at 15 nl/min with a hypotonic solution containing (in mM) 28 HCO_3, 26 Cl, 56 Na, 2 K, 1.8 Ca, 22 urea, and 4 gluconate, as well as 0.05% FD and C green dye no. 3 (Keystone, Chicago, IL) and 0.1% albumin. The perfused bicarbonate load, higher than in free flow, is chosen to more easily reveal the effect of inhibitors. Sample collections are quantitative and timed. A 10-min preperfusion period precedes all collections.

Groups of five-sixths nephrectomized rats are treated with various agents, e.g., angiotensin II, angiotensin$_1$ receptor antagonists, or Na^+/H^+ antiporter inhibitors.

Analyses

Whole blood and urine pH and P_{CO2} are measured quantitatively by an electrode blood-gas system and HCO_3 concentrations are calculated. Plasma and urine Na^+ and K^+ concentrations are measured by flame photometry and Cl^- concentrations by electrotitration. Plasma total protein concentrations and urine specific gravity are measured by refractometry, and hematocrits are determined by a microcapillary reader. Urine osmolalities are determined by freezing-point osmometry. Plasma creatinine concentrations are determined by the Jaffé method without deproteinization.

Perfusate and sample total CO_2 concentrations are measured by microcalorimetry (Levine et al. 1996). A standard curve is run before sample analysis, and standard samples bracket the determination of sample and perfusate CO_2 determination.

EVALUATION

The perfusion rate (R_P) is calculated as the product of the measured collection rate (R_C) and the ratio of inulin concentration in collected tubular fluid and perfusate. Water absorption (J_V) is calculated as the difference between the calculated perfusion rate and the measured collection rate (R_P minus R_C). J_{tCO_2} is calculated as

$$J_{tCO_2} = [(R_P C_P) - (R_C C_C) / L$$

whereby C_P and C_C are the measured CO_2 concentrations in perfusate and collected fluid, respectively, and L is the tubular length in millimeters, measured by dissection after latex injection.

Data are expressed as the means ±SE. Statistical significance is assessed by two-tailed unpaired Student's *t*-test or one way analysis of variance (ANOVA) followed by either Dunnett's test for multiple comparisons vs. control or the Newman-Keuls test for all pairwise comparisons. Tests indicating a value of $P < 0.05$ indicate a statistically significant difference between groups.

MODIFICATIONS OF THE METHOD

Function of the remnant kidney after subtotal (5/6) nephrectomy in **rats** has been used for many purposes:

to test the potential benefit of calcium antagonists (Tolins and Raij 1990; Jarusiripipat et al. 1992; Van den Branden et al. 1997) or antioxidants (Vaziri et al. 1998),

to verify the effect of ACE-inhibitors (Pelayo et al. 1990; Kakinuma et al. 1992; Ashab et al. 1995; Liu et al. 1996; Ali et al. 1998; Cohen et al. 1998; MacLaughlin et al. 1998), angiotensin antagonists (Kohzuki et al. 1994, 1995; Brooks et al. 1995; Barreto-Chaves and Mello-Aires 1996; Noda et al. 1997; Lariviere et al.

1998; Roczniak et al. 1999) and endothelin receptor antagonists (Nabokov et al. 1996; Potter et al. 1997; Wolf et al. 1999; Brochu et al. 1999; Shimuzu et al. 1999), or Na^+/H^+ antiporter inhibitors (Fernandez et al. 1994),

to study the influence of hormones, such as parathyroid hormone (Fukagawa et al. 1991; Urena et al. 1994; Yi et al. 1995; Schaefer et al. 1996), growth hormone (Santo et al. 1992; Garcia de Boto et al. 1996), insulin-like growth factor I (Hazel et al. 1994; Mak and Pak 1996; Tonshoff et al. 1997), vasopressin (Bardoux et al. 1999), atrial natriuretic factor (Wong and Wong 1991, 1992), Luk et al. 1995) or erythropoietin (Poux et al. 1995; Zhou et al. 1997), during development of chronic renal failure.

Kimura et al. (1999) reported a model of progressive chronic renal failure in *rats*, produced by a single injection of microspheres (20 to 30 µm in diameter) into the left renal artery after right nephrectomy.

Cowley et al. (1996) described the **Han:SRPD rat** strain which develops autosomal dominant polycystic kidney disease with chronic renal failure that resembles human autosomal dominant polycystic kidney disease.

Chronic renal failure can be induced by feeding a lithium containing diet (40–50 mmol/kg) to newborn *rats* until an age of 55–65 weeks (Christensen et al. 1992, 1997; Nyengaard et al. 1994).

Stockelman et al. (1998) described chronic renal failure in a **mouse** model of human adenine phosphoribo-syltransferase deficiency. Hamilton and Cotes (1994) used a partial nephrectomy model in *mice* with two-thirds of total renal mass excised to evaluate erythropoiesis and erythropoietin production from extrarenal sources such as the submandibulary salivary gland. Koumegawa et al. (1991) suggested the DBA/2FG-pcy *mouse*, which develops numerous cysts in kidney cortex and medulla, a progressive anemia and an elevation of blood urea nitrogen, as useful spontaneous model of progressive renal failure.

Brown et al. (1990) studied the metabolism of erythropoietin in normal and uremic **rabbits** with 5/6 nephrectomy. Bonilla-Felix used *rabbits* after 75% nephrectomy to study the response of cortical collecting ducts from remnant kidneys to arginine vasopressin.

Fine et al. (1990), Vaneerdeweg et al. (1992) described surgical techniques for kidney resection to produce chronic renal failure in **dogs**.

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C.2.3 Experimental nephritis

C.2.3.1 General considerations

Various experimental procedures were described as models for glomerulonephritis in human beings. Most of them were developed in rats and rabbits. They involve the reactions of antibodies against renal components, such as Masugi nephritis (Masugi and Sato 1934), Heymann nephritis (Heymann 1959), nephrotoxic serum nephritis (Unanue and Dixon 1967), crescentic type anti-glomerular membrane nephritis (Nagoe et al. 1994, 1998), anti-Thy1 nephritis (Chen et al. 1999).

Moreover, MRL Mpf lpr/lpr (MRL/lpr)-mice were described which spontaneously develop a severe disease with many symptoms very similar to human systemic lupus erythematodes, i.e. hypergammaglobulinemia, and glomerulonephritis (Theofilopoulos and Dixon 1981; see also I.2.2.16).

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C.2.3.2 Nephrotoxic serum nephritis

PURPOSE AND RATIONALE

Nephrotoxic serum nephritis is produced in animals by administration of heterologous antibody against glomerular basement membrane. This is regarded as an experimental model of human glomerular immune injury resulting in glomerulonephritis (Unanue and Dixon 1967). The glomerular lesions induced by nephrotoxic serum nephritis vary with species. The Wistar-Kyoto rat is susceptible to the induction of a crescentic glomerulonephritis following small doses of nephrotoxic serum (Kushiro et al. 1998; Suzuki et al. 1998).

PROCEDURE

Preparation of nephrotoxic serum

Normal Wistar rat kidneys are fully perfused with physiologic saline through a catheter placed in the aorta. Renal cortical tissue is removed, homogenized and diluted with physiological saline at about 20% suspension. Two ml of renal cortical homogenate are emulsified with an equal volume of Freund's complete adjuvant. This emulsion is injected subcutaneously into rabbits twice a month for two months. Seven days after the last injection, the rabbits are bled from the carotid artery under anesthesia. The sera are decomplemented for 30 min at 56 °C and absorbed with freshly harvested rat erythrocytes.

Experimental protocol

Male Wistar-Kyoto rats weighing 150 g receive either continuous administration of the test drug by an osmotic pump (ALZA Co., Palo Alto, USA) or saline. Twenty-four hours later, the rats are injected with 1 ml of nephrotoxic serum. At 9, 12, and 14 days, urine samples are collected and urinary protein levels are measured using the Lowry method. At 14 days the rats are sacrificed under ether anesthesia, and both kidneys are removed. Portions of these tissues are processed for light microscopy, immunofluorescence staining and immunoperoxidase staining.

For light microscopy, tissues are fixed and embedded in paraffin. Sections are stained with hematoxylin and eosin, and periodic acid Schiff's reagent. Twenty glomeruli are examined per rat and number of glomeruli which forms crescent is counted.

Indirect immunofluorescence studies are performed on 3 μm-thick cryostat sections which are air dried and incubated with anti-rat intercellular adhesion molecule-1 (ICAM-1) antibody (Tamatani and Miyasaka 1990) for 60 min at room temperature. After washing the

antibody binding is visualized by incubating the sections for 30 min with fluorescein isothiocyanate-labeled goat anti-mouse IgG.

Direct immunofluorescence studies are performed on 4 µm thick cryostat sections, which are incubated with fluorescein isothiocyanate-labeled goat anti-rat IgG, goat anti-rat C3, goat anti-rat fibrinogen and goat anti-rabbit IgG.

The staining intensity of 20 glomeruli per rat is semiquantitatively assessed into 4 grades.

For **immunoperoxidase staining** the distribution of leukocytes is examined using an immunoperoxidase ABC kit (Vector Lab, Burlingame, USA). Nonspecific protein binding is blocked by incubating the cryostat sections with 10% bovine serum in Tris-buffered saline for 20 min. Nonspecific staining is blocked by 15 min incubation with avidin and then biotin using the avidin-biotin blocking kit (Vector Lab). Endogenous peroxidase activity is inhibited by incubating the sections in methanol containing 0.3% H_2O_2 for 20 min. Sections are first incubated for 60 min with primary antibodies at room temperature, incubating monoclonal antibodies against rat monocytes/macrophages (ED-1), rat CD4 and rat CD8. Then the sections are incubated with biotinylated donkey anti-mouse IgG for 30 min at room temperature. Biotinylated horseradish peroxidase is applied for 30 min at room temperature. Peroxidase activity is developed in 3,3-diaminobenzidine and hydrogen peroxide. The sections are then counterstained with Mayer's hemato-xylin. The number of ED-1 positive cells, CD4 positive cells and CD8 positive cells per glomerular cross-section is counted in 20 glomeruli per rat.

EVALUATION

All data are expressed as mean ±SEM. Significance of differences between groups is determined using Wilcoxon's test.

MODIFICATIONS OF THE METHOD

Masugi and Sato (1934), Krakower and Greenspon (1951), Heyman et al. (1959, 1965), Eddington et al. (1968) already described experimental allergic glomerulonephritis in rats.

Ito et al. (1983), Nagao et al. (1994, 1998) induced crescentic type antiglomerular basement membrane nephritis in male Sprague Dawley rats by injecting 6.5 mg rabbit gamma-globulin in 0.25 ml Freund's complete adjuvant into the hind foot pads, following the injection of 0.6 ml of rabbit anti-rat glomerular basement membrane serum into the tail vein.

Couser et al. (1978) studied the development of immune deposits on the subepithelial surface of the glomerular capillary wall in isolated rat kidneys perfused at controlled perfusion pressure, pH, temperature, and flow rates with recirculating oxygenated perfusate containing bovine serum albumin in buffer and sheep antibody to rat proximal tubular epithelial cell brush border antigen.

Hayashi et al. (1996) tested the effects of a flavonoid in original-type anti-glomerular basement membrane antibody associated glomerulonephritis in male Sprague Dawley rats on upregulation of intracellular adhesion molecule expression and on increase in leukocyte function-associated antigen positive cells in nephritic glomeruli.

Nagamatsu et al. (1999) found beneficial effects of an angiotensin II type I receptor antagonist in anti-glomerular basement membrane antibody-associated nephritis in rats.

Sanaka et al. (1997) evaluated the effects of a free radical scavenger on the progression of nephrotoxic serum nephritis in male Sprague Dawley rats. The rat glomerular basement membrane was prepared according to the method of Krakower and Greenspon (1951) who localized the nephrotoxic antigen within the isolated renal glomerulus.

Kawasaki et al. (1992) induced crescentic glomerulonephritis with a small dose of nephrotoxic serum in WKY rats, which was characterized by the early infiltration of CD8 positive cells in glomeruli. *In vivo* depletion of CD8 positive cells completely prevented proteinuria and crescent formation.

Okuda et al. (1990) provided evidence of an elevated expression of transforming growth factor-β, proteoglycans and fibronectin in glomerulonephritis induced in rats by injection of anti-thymocyte serum.

Hamada and Nagase (1996), Chen et al. (1999) induced anti-Thy1 nephritis with the antibody to the Thy-1 antigen which is present in the mesangial cells of the glomeruli. The early pathobiological cellular events are characterized by invasion of platelets, polymorphonuclear leukocytes, and monocytes into the glomerulus which occurs within hours after induction of nephritis. Complement-dependent mesangiolysis then ensues between day 1 and 3.

Passive Heymann nephritis (Heymann 1959) was used a model by Hara et al. (1991), Nagao et al. (1996), Heise et al. (1998). The disease is induced in rats by heterologous antibody to crude renal border antigen Fx1A. The model is characterized by granular deposition of heterologous and homologous antibody and complement along the glomerular capillary wall and as a counterpart, extensive electron-dense subepithelial deposits are seen at the ultrastructural level. Massive proteinuria develops after a latent period of 2 to 4 days in the absence of glomerular hypercellularity.

Kawasaki et al. (1995) studied the therapeutic effect of combined treatment with monoclonal antibodies against intercellular adhesion molecule 1 (ICAM-1) and lymphocyte-function-associated antigen 1 (LFA-1) in **Masugi nephritis** of Wistar-Kyoto rats.

Thaiss et al. (1989) evaluated the effect of the immunosuppressant cyclosporin A on an active model of *in situ* immune complex glomerulonephritis. Wistar rats were preimmunized with human IgG and 2 weeksafter the last antigen injection, the left kidney was perfused with cationized human IgG in order to induce unilateral *in situ* immune complex glomerulonephritis.

Okubo et al. (1990) studied the immunosuppressive effects of FK506 on active Heymann's nephritis and the autologous phase of Masugi nephritis.

Rennke et al. (1994) developed a model system of acute nephritis in the rat whereby a chemically reactive form of the hapten azobenzenearsonate is introduced directly in to the left kidney of pre-immunized Brown Norway rats.

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C.2.4 Experimental nephrosis

PURPOSE AND RATIONALE

Some compounds used in antineoplastic therapy induce nephrosis in animals, like the antibiotic adriamycin (= doxorubicin) (Milner et al. 1991, 1994; Chagnac et al. 1994; Wapstra et al. 1996; De Boer et al. 1999; Mutti et al. 1999) or daunomycin (Kimura et al(1993), the aminonucleoside puromycin (Yayama et al. 1993; Guoji et al. 1994; Magil 1996; Ebihara et al. 1997; Nosaka et al. 1997; Park et al. 1998; Asami et al. 1999; Pedraza-Chaverri et al. 1999) or cisplatin (Abdel-Gayoum et al. 1999).

These models allow to test the protective effects of drugs even after the renal disease in established, i.e. mimic the clinical situation (Wapstra et al. 1996).

PROCEDURE

Male Wistar rats with an initial weight of about 300 g receive a single intravenous dose of 2 mg/kg adriamycin. Twice a week during a 12-weeks period, the animals are weighed, 24-h urine is collected and blood pressure is measured by the tail-cuff method.

During the first 5 weeks, all animals are kept on a low sodium diet with tap water ad libitum. After stabilization of proteinuria (5 weeks), animals are divided into 2 groups receiving either low sodium or normal sodium diet. After a week stabilization on these diets, animal receive different doses of treatment in their drinking water. These regimen is continued until the end of the study (week 12), at which time all animals are sacrificed and blood samples and kidney tissue are obtained.

During each blood pressure measurement session, five measurements are recorded for each animal. The blood pressure is taken as the mean of the last 3 recordings. Urinary protein is determined by the Pyrogallol Red-molybdate method (RA-1000 Technicon). Urinary sodium, creatinine and urea and serum electrolytes, creatinine, albumin, cholesterol and triacylgly-cerols are measured by a standard autoanalyser technique. Kidney samples are fixed in paraformaldehyde and embedded in paraffin. Sections are stained with the periodic acid/Schiff technique. Focal glomerular sclerosis is scored semiquantitatively by light microscopy.

EVALUATION

Analysis of co-variance is used to compare the experimental groups (defined by diet or dose of drug) after 2 weeks of treatment (week 8) and the end of the study (week 12) with the baseline value (week 6) as a co-variable. Tuckey's method is used for comparison of groups receiving different doses of drug. The other statistical comparisons are performed using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Fawn-Hooded rats develop systemic hypertension and spontaneous age-dependent glomerulosclerosis with proteinuria (Mackenzie et al. 1997).

Spontaneous nephrosis with proteinuria occurs in Dahl salt-sensitive rats fed on a normal sodium diet (Yoneda et al. 1998).

Mizuno et al. (1999) studied the ICGN mouse strain as a unique model for naturally occurring nephrotic syndrome.

Focal segmental glomerulosclerosis with heavy proteinuria has been found in mice in which the Mpv17 gene was inactivated (Mpv17 –/– mice). Binder et al. (1999) recommended these animals as model of steroid-resistant nephrosis sensitive to radical scavenger therapy.

Kimura et al. (1993) described strain specificity in the susceptibility of mice to daunomycin-induced nephrosis.

Klahr and Morrissey (1997) described the effects of ACE inhibitors and angiotensin II receptor antagonists on various parameters associated with renal interstitial fibrosis induced by unilateral ureteral obstruction in rats.

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C.3
Uricosuric and hypo-uricemic activity

C.3.1
In vitro methods

C.3.1.1
Inhibition of xanthine oxidase *in vitro* indicating hypouricemic activity

PURPOSE AND RATIONALE

Xanthine oxidase catalyzes the oxidation of hypoxanthine and xanthine to uric acid. Xanthine oxidase is a complex metalloflavoprotein containing one molybdenum, one FAD and two iron-sulfur centers of the ferredoxine type in each of its two independent subunits. Usually, the enzyme is isolated from cow's milk. The enzyme is inhibited by allopurinol and related compounds. The production of uric acid from the substrate (xanthine) can be determined by measuring the change in optical density in the UV range.

PROCEDURE

The test compound is incubated with xanthine oxidase (usually derived from milk, sometimes derived from rat liver or small intestine), EDTA and phosphate buffer solution (pH 7.8) at 37 °C. Control solutions without test compound are incubated under identical conditions. Following addition of xanthine, the change in absorbance is determined.

Assay conditions:
- wavelength: 293 nm
- line path: 10 mm
- final volume: 1.0 ml

EVALUATION

The percent inhibition of xanthine oxidase is determined relative to control solutions.

IC_{50} values of test compounds are calculated.

Standard data:
- Allopurinol: IC_{50}: ca 10^{-8} mol/l

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C.3.1.2
Urate uptake in brush border membrane vesicles

PURPOSE AND RATIONALE

The urate-anion exchanger system in brush border membrane vesicle, which mediates hydroxyl ion gradient-dependent urate uptake, is the most likely route for the mediation of urate transport in the first step of urate reabsorption in the proximal tubules. Luminal drugs which inhibit urate reabsorption are inhibiting the transport of urate by blocking the urate/anion exchanger.

PROCEDURE

Male Sprague Dawley rats weighing 320–380 g are sacrificed by decapitation and the kidneys are removed immediately. All steps for the preparation of brush border membrane vesicles are carried out at 4 °C. Renal

cortex is homogenized for 2 min in a medium containing 250 mmol/l mannitol, 10 mmol/l Tris, 16 mmol/l HEPES buffer (pH 7.5) using a Physcotron homogenizer. The homogenate is centrifuged at 2 400 *g* for 10 min and the supernatant is centrifuged at 2 800 *g* for 20 min. Subsequently, the supernatant is discarded and the loosely packed membrane-rich layer is flushed off from the bottom densely packed brown pellet. The membrane-rich layer is resuspended manually in 250 mmol/l mannitol containing 10 mmol/l Tris-HEPES (pH 7.5) using a Dounce homogenizer and $MgSO_4$ is added to a final concentration of 10 mmol/l. After standing for 20 min, the suspension is centrifuged at 2 400 *g* for 20 min and the supernatant containing brush border membranes is recentrifuged two more times at 2 400 *g* for 20 min. The final supernatant is centrifuged at 28 000 *g* for 20 min and the pellet is suspended in a small amount of medium containing 150 mmol/l mannitol, 50 mmol/l potassium phosphate buffer (pH 7.5) and 2 mmol/l $MgSO_4$ to a final protein concentration of 4–8 mg/ml. The brush border membrane vesicle preparation is frozen and stored at –80 °C until use.

After preincubation of the brush border membrane vesicle preparation for 2 h, [2-^{14}C]urate uptake is initiated by adding 200 µl of incubation medium to 20 µl of the membrane suspension. The incubation medium has the following composition (mmol/l): 150 mannitol, 2 $MgSO_4$, 50 potassium phosphate buffer, pH 6.0 or 7.5, 0.02 [2-^{14}C]urate, and various concentrations of the inhibitor. At 10 s after the addition of the incubation medium, 200 µl portions of the suspension are pipetted onto the center of prewetted cellulose acetate filters kept under suction. The vesicles retaining on the filter are washed immediately with 5 ml of an ice-cold solution containing 150 mmol/l mannitol and 50 mmol/l potassium phosphate buffer, pH 6.0 or 7.5, which is used at the same pH as the incubation medium. Preincubations and incubations are performed at 23 ±1 °C. Each experiment is performed in triplicate. Corrections are made for the radioactivity bound to the filters in the absence of membrane vesicles. The term of the OH^- gradient-dependent urate uptake is defined as the difference between the uptakes in the incubation medium at pH 6.0 and that at pH 7.5. The OH^- gradient-dependent urate uptake at 10 s is assumed to present an initial velocity.

EVALUATION

From a concentration-response curve relating log concentration of drug to the logit activity of the OH^- gradient-dependent urate uptake for 10 s, IC_{50} (concentration producing 50% of inhibition) is determined by least-squares regression analysis.

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C.3.2 *In vivo* methods

C.3.2.1 Diuretic and uricosuric activity in mice

PURPOSE AND RATIONALE

Renal excretion of uric acid consists of three components: complete filterability of uric acid in the glomerulus, subsequent tubular reabsorption, and tubular secretion (Gutman and Yü 1961). Pronounced species differences have been described in uric acid metabolism including man. Mice were recommended for primary screening of uricosuric drugs.

PROCEDURE

Male NMRI mice weighing 25–30 g are used. On the evening prior to the experiment, food but not water is withheld. In the morning, the mice are orally loaded with 50 ml/kg 0.9% NaCl-solution. Together with the sodium load the test compound is applied by gavage in 2% starch suspension. Controls receive saline and starch suspension only. Groups of 5 mice are placed into metabolism cages. Urine is collected over 4 h. In the urine sodium and potassium are determined by flame photometry, chloride by argentometrically with potentiometrical end point titration (Chloride titrator, Aminco®), uric acid by the Uriquant®-method, creatinine by the Jaffé-reaction, as well as pH and osmolality.

EVALUATION

Urine excretion is calculated in ml/kg. Uric acid-, creatinine- and ion- excretions are calculated in mmol/kg and expressed as percent changes versus controls. The changes are evaluated statistically using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

Some saluretic-diuretic agents, like ethacrynic acid, are inactive in the rat, when given orally. Moreover,

uricosuric activity in mice is less reliable than that in primates.

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C.3.2.2 Hypouricemic activity after allantoxanamide treatment in rats

PURPOSE AND RATIONALE

Most species used for pharmacological experiments have rather low blood levels of uric acid. Experimental hyperuricemia can by induced by inhibition of the enzyme uricase. In most species uricase metabolizes uric acid to allantoin. Allantoxanamide blocks uricase and increases endogenously synthesized uric acid. This increase is blocked by compounds like allopurinol.

PROCEDURE

Non fasted male Sprague Dawley rats weighing 230–280 g are treated by intraperitoneal injection of 250 mg/kg allantoxanamide suspended in 5 ml/kg sesame oil. The test compound is applied orally in a dose of 50 mg/kg in 40 ml/kg water. Likewise, the standard compound allopurinol is given in a dose of 50 mg/kg. Eight rats are used for the each dose of test drugs and standard. The animals are placed individually into metabolism cages with free access to food and water. Urine is collected during the periods of 1 to 6 and 7 to 24 h. Blood is withdrawn by retroorbital puncture prior and 2, 6 and 24 h after compound administration. Uric acid is determined with the Uric-aquant®-method in plasma [mmol/l] and urine [mmol/l].

EVALUATION

Mean values of uric acid concentrations in plasma at the different time intervals and mean values of uric acid excretion after 6 and 24 h of the test group are compared with the control group (allantoxanamide treated only) using Student's *t*-test.

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C.3.2.3 Hypouricemic and uricosuric activity after potassium oxonate treatment in rats

PURPOSE AND RATIONALE

Increase of uric acid in serum of rats is induced by a special diet and the uricase inhibitor potassium oxonate. Uric acid concentration in serum and uric acid excretion in urine prior and after three experimental days are determined.

PROCEDURE

Male Wistar rats weighing 250 g are placed individually in metabolic cages. They are offered a special diet containing 5% fructose, 3% uric acid, 2% potassium oxonate (2,4-dihydroxy-1,3-triazine-6-carbonic acid) and 0.001% artificial sweetener. Drinking water consists of a 0.5% solution of potassium oxonate. The animals are treated orally with 50 or 100 mg/kg of the test compound or the standard (allopurinol) dissolved in 5 ml/100 g body weight of 0.5% potassium oxonate solution. The treatment is repeated on the second day. On the third day 24 h urine is collected and the animals are sacrificed by exsanguination. Concentrations of uric acid and electrolytes (Na^+, K^+, Cl^-) are determined in blood and urine.

EVALUATION

Concentrations of uric acid and electrolytes in blood and urine of animals treated with the test compound are compared statistically with control and standard drug treated animals.

MODIFICATIONS OF THE METHOD

Clearance techniques in oxonate-treated rats were used by Yonetani et al. (1987), Shinosaki et al. (1991), Dan et al. (1994).

Sugino and Shimada (1995) tested uricosuric effects in oxonate-loaded rats, in the pyrazinoic acid suppression test and in the phenolsulfonphthalein test.

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C.3.2.4 Phenol red excretion in rats

PURPOSE AND RATIONALE

Phenol red (= phenolsulfonphthalein) excretion is an indirect test for uricosuric activity. After intravenous injection phenol red is mainly eliminated by active secretion in the proximal tubulus of the kidney. Treatment with uricosuric agents decreases the secretory activity of tubulus cells resulting in a delayed excretion of phenol red. Plasma values of phenol red are increased in treated animals as compared to controls.

PROCEDURE

Male Wistar rats weighing 120–150 g are treated orally with the test compound or the standard 30 min prior to intravenous injection via the tail vein with 2.5 ml/kg of a 3% aqueous solution of phenolsulfonphthalein. For intravenous application, 5.0 ml/kg of the test drug solution are injected immediately after the phenolsulfonphthalein injection followed by flushing with 2.5 ml/kg saline. By retro-orbital puncture blood samples are with-drawn after 30, 60 and 180 min. Blood (0.2 ml) is diluted with 2 ml 0.9% NaCl-solution and centrifuged. To 1 ml of the supernatant 1 ml of 1% sodium carbonate solution and 8 ml of saline are added. Using a spectrophotometer (Eppendorf, Hamburg) extinction at 546 nm is determined.

EVALUATION

Extinction values are calculated for total blood. At each time interval the values in treated rats are compared statistically with those of controls.

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C.3.2.5 Uricosuric activity in Dalmatian dogs

PURPOSE AND RATIONALE

Most species have low plasma levels and low renal excretion of uric acid. The Dalmatian dog is an exception with excessive uric acid excretion, but with relatively high plasma levels. Explanations are a genetically determined defect in tubular reabsorption of the filtered urate (Friedman and Byers 1948; Kessler et al. 1959) and a defective hepatic uricase activity (Yü et al. 1971). The Dalmatian dog is being used for studies of uricosuric agents.

PROCEDURE

Conscious male Dalmatian dogs with a body weight of about 20 kg are used. Food but not water is withheld 24 h prior to the experiment. The animals are placed individually in metabolic cages. The urinary bladder is emptied by catheterization. Twenty ml/kg drinking water is applied by gavage. Every 2 h the animals are catheterized again, blood is withdrawn from a jugular

vein and additional 8 ml/kg drinking water is applied by gavage. The urine and blood values obtained after the first 2 h serve as control. Then, the test compound is applied either i.v. or orally. Up to 8 h blood and urine samples are collected every 2 h. No water is given after the last sampling. The dogs stay over night in the metabolic cages. Twenty-four hours after beginning of the experiment, venous puncture and catheterization is performed once more. Blood and urine samples are analyzed for uric acid (Uricaquant®-method), creatinine (Jaffé reaction), sodium and potassium (flame photometry), calcium and magnesium (atom absorption method), and chloride (argentometry) as well as for osmolality.

EVALUATION

The values after application of the drug are compared with predrug values.

CRITICAL ASSESSMENT OF THE METHOD

Dalmatian dogs bred by commercial breeders are not always homozygous. Therefore, not every dog is suitable for experiments on uric acid excretion.

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C.3.2.6 Uricosuric activity in Cebus monkeys

PURPOSE AND RATIONALE

The Cebus monkey is described not to possess uricase (Simkin 1971) and to have different metabolic conditions for uric acid than other experimental animals. This species is chosen for special studies of anti-uricopathic drugs since it resembles more closely human uric acid metabolism.

PROCEDURE

Cebus monkeys (*Cebus albifrons*) of either sex weighing 3.0 to 5.0 kg are used. Twenty-four h prior to the test, food is withheld, but water is available ad libitum. On the morning of the experiment, the animals receive 20 ml/kg drinking water by gavage, followed by oral application of the test compound. Control animals receive water only. The animals are placed in individual metabolism cages and the spontaneously voided urine is collected after 2, 6, and 24 h. After 2 and 6 h, additional 4 ml/kg water is given by gavage. From a cubital vein blood is withdrawn prior to the experiment and 2, 6 and 24 h after application. Urine and serum samples are analyzed for uric acid (Uricaquant®-method), creatinine (Jaffé reaction), sodium and potassium (flame photometry), calcium and magnesium (atom absorption method), and chloride (argentometry) as well as for osmolality.

EVALUATION

Allopurinol and probenicide are used as standard drugs and are compared with test compounds.

CRITICAL ASSESSMENT OF THE METHOD

The use of the Cebus monkey as animal model has been proven to be the most valuable method to test putative hypouricemic compounds (Hropot 1988).

MODIFICATIONS OF THE METHOD

Onuma et al. (1988) used Cebus monkeys for evaluation of uricosuric effects of an aryloxyacetic derivative.

Yonetani et al. (1987) performed clearance experiments with uricosuric drugs in anesthetized chimpanzees.

Dan et al. (1989) tested the activity of AA-193, an uricosuric agent in rats, mice and Cebus monkeys.

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C.4 Influence on lower urinary tract

C.4.1 *In vivo* studies

C.4.1.1 Micturition studies

PURPOSE AND RATIONALE

Urinary incontinence is a major psychosocial, medical, and economic problem. The most common condition to be treated pharmacologically is incontinence due to detrusor instability. The response of the urinary bladder to filling with increasing volumes of fluid (cystometrogram) is a common procedure for evaluating bladder function in both animals and humans. The response of the vesico-urethral complex can be arbitrarily divided into the collection and expulsion phases. The nervous control of the detrusor, the internal and the external sphincter has been reviewed by Kuru (1965). A detailed description of the nervous control of the urinary bladder of the cat has been given by De Groat (1975). The pharmacology of lower urinary tract muscles and penile erectile tissues has been reviewed by Anderson (1993). Ferguson and Christopher (1996) reviewed urine bladder function and drug development. Urine storage and timely expulsion of bladder content are produced through the coordinated activation of a series of reflexes involving cholinergic, sympathetic, and, possibly, purinergic, serotonergic, and peptidergic innervation. In view of this complexity, *in vivo* models were developed for the quantitative analysis of the effects of drugs on the function of the vesico-urethral complex (Maggi et al. 1983, 1985, 1986, 1987a,b, 1992).

PROCEDURE

Male Wistar rats weighing 340–360 g are anesthetized by subcutaneous injection of 1.2 g/kg urethane. The left jugular vein is cannulated for drug injection. Body temperature is kept constant by means of a heating pad maintained at 37 °C. Through a midline incision of the abdomen, the urinary bladder is exposed and emptied of urine by application of a slight manual pressure. A 20-gauge needle is inserted through the apex of the bladder dome for 3–4 mm into its lumen. The needle is connected to a pressure transducer by means of a polyethylene tubing (1.5 mm OD and 1.0 mm ID) and the whole system filled with saline. The tubing is provided with an internal coaxial polyethylene tubing (0.6 mm OD and 0.3 mm ID) inserted through a side hole and sealed by a drop of epoxy resin. The second tubing serves for intravesical infusion of fluid and is connected, through a peristaltic pump, to a saline reservoir.

Intraluminal pressure signals are delivered to an amplifier and displayed on a four channel polygraph. Warm saline-soaked cotton wool swabs are laid around the exteriorized organ to maintain its temperature and to keep it moist in experiments involving the topical application of substances on the bladder dome.

After a 15-min equilibration period at zero volume, variations in intraluminal pressure are recorded in response to continuous infusion of saline at a rate of 2.8 ml/h at 37 °C for 30–40 min by means of a peristaltic pump connected to the polyethylene tubing inserted into the bladder. This infusion rate simulates the maximal hourly diuresis within the physiological range. In each preparation the infusion is continued until micturition occurs. Micturition is referred as the emission of several drops of fluid during a sustained phasic contraction of the detrusor muscle which is followed by return to zero or, in any case, to a value lower than that recorded just before micturition.

For both intravenous and topical administration, substances are dissolved in saline.

EVALUATION

In each experiment, the following parameters are evaluated:

1. pressure threshold = intraluminal pressure value recorded just before micturition
2. volume threshold = the volume of infused saline required to obtain micturition
3. maximal amplitude of micturition contraction
4. residual volume after micturition

The effect of substances on the compliance of the bladder wall is evaluated by comparing the volume-pressure-relationship of treated animals with that of controls.

Statistical analysis of the data is performed by means of the Student's *t*-test for paired or unpaired data, or by means of analysis of variance followed by the Tukey test. Statistical analysis of nonparametric data is made by the chi square test.

MODIFICATIONS OF THE METHOD

Either chemical (6-hydroxydopamine, reserpine) or surgical (section of hypogastric nerves) sympathectomy produces a picture of detrusor hyperreflexia and urine dropping, mimicking cystometric finding in human disease (Maggi et al. 1987a).

Postius and Szelenyi (1983) described a model for *in vivo* screening of spasmolytic compounds using the rat bladder.

Dray (1985) used the spontaneous, volume-induced contractions of the urinary bladder in the anesthetized rat to assess the central activity of substances with opioid properties.

Pietra et al. (1990) studied the effects of some antidepressants on the volume-induced reflex contractions of the rat urinary bladder. The urinary bladder of anesthetized rats was filled via the recording catheter by incremental volumes of warmed saline until bladder contractions occurred as a result of central activity. Volume-induced contractions were then recorded and occurred rhythmically and reproducibly for 2–3 h. Drug activity was assessed in each animal against the background frequency of bladder contractions, for a 15-min time period following intravenous administration of different doses.

Harada et al. (1992) proposed a method for rapid evaluation of the efficacy of pharmacologic agents and their analogs in enhancing bladder capacity and reducing the voiding frequency. Conscious rats were placed in a restrainer over a urine collector. The collector was secured to an Statham UC3 strain transducer, the output of which was amplified by a Gould bridge amplifier. Data were monitored on a polygraph.

Conte et al. (1991) proposed a method for simultaneous recording of vesical and the external urethral sphincter pressure in urethane-anesthetized rats.

Angelico et al. (1992) reported *in vivo* effects of different antispasmodic drugs on the rat bladder contractions induced by topically applied KCl.

Oyasu et al. (1994) measured spontaneous bladder contractions caused by raising the intravesical volume in anesthetized rats.

Yaksh et al. (1986) described a chronic model for study of micturition in unanesthetized rats. A bladder catheter was implanted chronically through laparatomy and externalized percutaneously.

Horváth et al. (1994) reported an ultrasonic method to study the influence of drugs on micturition in intact rats.

Tillig and Constantinou (1996) described videomicroscopic imaging of urethral peristaltic function in anesthetized rats. Cystometrograms were performed by recording continuously the bladder pressures while detecting micturition using a sensor placed at the orifice of the urethra. Renal pelvic pressure was measured during continuous perfusion using a nephrostomy inserted through the parenchyma. A catheter was placed in the femoral vein for intravenous drug administration. The left pyelo-ureteric junction and the upper part of the ureter were visualized using a stereomicroscope equipped with a video camera and a tape recorder. One syringe pump was used for filling the bladder to perform continuous cystometrograms. An other syringe pump was used for infusion of indigo carmine to assist the visualization of the bladder pressure.

Conte et al. (1988) developed a cystometric technique for quantitative studies on physiopharmacology of micturition in conscious, freely moving rats.

Peterson et al. (1989), Noronha-Blob et al. (1991) described *in vivo* cystometrogram studies in urethane-anesthetized and conscious **guinea pigs**.

Moreau et al. (1983) described simultaneous cystometry and uroflowmetry for evaluation of the caudal part of the urinary tract in **dogs**.

Imagawa et al. (1989) reported an *in vivo* procedure for functional evaluation of sympathetically mediated responses in lower urinary tract of dogs.

Häbler et al. (1990, 1992) examined the functional properties of unmyelinated and myelinated primary afferent neurons innervating the pelvic viscera in anesthetized **cats**. The axons were isolated from the intact dorsal root and the intact or chronically de-efferented ventral root of segment S2. The responses of the neurons were studied with natural stimulation of the urinary bladder using innocuous or noxious increases of intravesical pressure.

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C.4.2
Studies in isolated organs

C.4.2.1
Studies on renal pelvis

PURPOSE AND RATIONALE

The isolated renal pelvis of the guinea pig has been used as pharmacological tool mainly by the group of Maggi (Maggi and Giuliani 1991, 1992; Maggi et al. 1992a,b, 1992c, 1994, 1995; Giuliani and Maggi 1996; Santicioli et al. 1995, 1997; Patachini et al. 1998; Bigoni et al. 1999).

PROCEDURE

Male albino guinea pigs weighing 250–300 g are stunned and bled. The whole kidney and attached ureter are removed and placed in oxygenated Krebs solution. The renal pelvis is carefully dissected from the renal parenchyma, separated from the ureter, cut and connected to threads to record motility along the circular axis. The preparation is suspended in a 5 ml organ bath and mechanical activity recorded by means of an isotonic transducer (load 1 mN). Transmural electrical field stimulation is made by means of platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a GRASS S 88 stimulator. Square wave pulses (pulse width 0.5 ms, 60 V) are delivered in trains of 10 s duration at frequencies of 5 to 10 Hz.

Experiments commence after a 60 to 90 min equilibrium period after which amplitude and frequency of spontaneous activity has reached a steady state. *In vitro* capsaicin desensitization is made by exposure of the preparation to 10 µM for 15 min, followed by washing out and further equilibration for 30–60 min.

Concentration response curves to noradrenaline and acetylcholine are performed by non-cumulative addition to the bath at 20 min intervals. Contact time of drugs is 15 min.

EVALUATION

All values are expressed as mean ±SEM Statistical analysis is performed by means of Student's *t*-test for paired or unpaired data or by analysis of variance.

MODIFICATIONS OF THE METHOD

Zhang and Lang (1994), Lang et al. (1995), Lang and Zhang (1996) Teele and Lang (1998) recommended circumferentially cut strips from the proximal renal pelvis of guinea pigs since these strips contract more frequently than strips cut from the mid region.

Kimoto and Constantinou (1990, 1991) studied contractility of smooth muscle strips from the pace-

maker regions and pelviureteric junction of renal pelvis from **rabbits**.

Kondo et al. (1992) determined the effects of dobutamine and terbutaline on adenylate cyclase activity and cyclic AMP content in the renal pelvis of rabbits.

Seki and Suzuki (1990) made intracellular recordings to study the electrical properties of smooth muscle cells in the rabbit renal pelvis.

Zwergel et al. (1991) developed an intact **canine** model to measure renal pelvic pressure after complete ureteral obstruction with a balloon catheter inflated in the distal ureter.

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C.4.2.2 Studies on urinary bladder and internal urethral sphincter

PURPOSE AND RATIONALE

Several authors investigated the influence of drugs on isolated parts of the lower urinary tract. Ueda et al. (1984) studied the effects on smooth muscle of the rabbit bladder dome, trigone and proximal urethra.

PROCEDURE

Male New Zealand rabbits weighing 2–3 kg are sacrificed and the abdomen is opened to remove the bladder and the urethra. After excess fat and connective tissue is removed, the bladder and the urethra are dissected into dome, trigone and proximal urethral preparations. All strips are cut transversely being approximately 2×6 mm unstretched. Ligatures are placed on both ends of the strips and one end is attached to a tissue holder and the other to a strain gauge force-displacement transducer connected to a polygraph on which isometric tension changes are recorded. Each of the strips is then placed into a 20 ml tissue bath containing Krebs-Ringer solution bubbled with 95% O_2 + 5% CO_2 at 37 °C. Resting tension is adjusted

to 1 g during an equilibrium period of at least 2 h. The contractile and relaxant responses are measured as increases or decreases from the resting tension. Doses-response curves are performed in a cumulative matter. Tissues are pretreated with an antagonist 20 min before the addition of an agonist.

EVALUATION

The values are expressed or plotted as the means ±SE and pA_2 values are calculated according to Arunlakshana and Schild (1959). Data are analyzed using the *t*-test, analysis of variance, Dunnett's test and regression analysis.

MODIFICATIONS OF THE METHOD

Pietra et al. (1990) studied the effects of some antidepressants by the *in vitro* inhibition of carbachol-induced contractions of rat detrusor strip preparations. The detrusor muscle tissue (bladder dome) was cut in a semicircular direction and further dissected into strip preparations measuring approximately 2×20 mm.

The preparation of the isolated, innervated urinary bladder of the rat was reported by Hukovic et al. (1965). Electrical stimulation was performed by a bipolar electrode from the nerves running close to the ureter.

Mapp et al. (1990) used the isolated rat urinary bladder to study the pharmacological modulation of the contractile response to toluene diisocyanate.

Maggi et al. (1985) used isolated detrusor strips of rat bladder connected to an isometric strain gauge and stimulated by field stimulation.

Anderson (1978) recommended the **rabbit** detrusor muscle a an unique in-vitro smooth muscle preparation. Rabbit detrusor muscles are thin and devoid of underlying submucosal tissue with parallel fiber orientation. The tissue exhibits autorhythmicity, characteristic of most single unit type smooth muscle preparations and can be employed in either isometric or isotonic organ bath recording systems.

Honda and Nakagawa (1986) studied the effects of the optical isomers of an alpha-1 adrenoceptor antagonist in rabbit lower urinary tract and prostate.

Khanna et al. (1977, 1981) evaluated the *in vitro* responses of three segments of rabbit lower urinary tract, e.g., the bladder body, the bladder base and the proximal urethra.

Ferguson and Marchant (1995) studied the inhibitory actions of GABA on rabbit urinary bladder muscle strips.

Andersson et al. (1983) studied the electrically induced relaxation of the noradrenaline contracted isolated urethra from rabbit and man. In rabbits, two circular transverse sections, each 4 mm long, were taken from the middle and upper parts of the urethra. Human urethral preparations were obtained from male patients undergoing cysto-urethrectomy en bloc because of bladder cancer. Rings of tissue were taken from the membranous and supra- and infra-collicular parts of the prostatic urethra.

Andersson et al. (1992) used transversal strips from the middle and upper part of rabbit urethra to study the involvement of nitric oxide in the electrically-induced, nerve-mediated relaxation.

Weetman (1972) described the preparation of the isolated, innervated urinary bladder in **guinea pigs**. Contractions of the tissue induced by nerve stimulation could be blocked by local anesthetics and by tetrodotoxin.

Isolated innervated, rat and guinea pig hemi-urinary bladder preparations were described by Dhattiwala and Dave (1975).

Burnstock et al. (1978) used recorded isometric tension of mucosal-free strips of the detrusor of the bladder from guinea pigs *in vitro* after electrical field stimulation.

Von Heyden et al. (1997) tested urethral relaxation after electrostimulation in guinea pigs. Male Hartley guinea pigs weighing 350–450 g were sacrificed and bladder, urethra and penis were dissected out. From each animal 4–6 urethral rings 1–2 mm thick were cut. The urethral rings were mounted according to their anatomical order: in channel 1 the most proximal ring (near the bladder neck) and in channel 6 the most distal ring (near the penile crura). The urethral rings were stretched by two spring-wire clips (Harvard Apparatus, South Natick, MA) whose tips closed in the urethral lumen. The manner in which the urethral rings were cut and mounted ensured that only the circularly oriented, mostly striated fibers contributed to the tension measured. Detrusor muscle was cut as a horizontal ring proximal to the trigone and mounted in the same way. The clips were connected with 4-O silk to a glass tissue support hook on one side and to an isometric force transducer (Föhr Medical Instruments GmbH, D-64342 Seeheim, Germany) on the other. A double-chambered bath (Föhr Medical Instruments GmbH, D-64342 Seeheim, Germany) was used in which the working chamber was connected to a second chamber, in which the gas (95% O_2 and 5% CO_2) was fed. The gas flow induced fluid circulation. Forces lower than 0.1 g could be measured without bubble artifacts in the working chamber. The transducer signals were fed into a thermal array recorder (Dash 10, Astro-Med, 63110 Rodgau, Germany). For tissue stimulation, vertical, L-shaped custom-made platinum electrodes (20 mm long, 0.3 mm diameter) 10 mm apart were used with a custom-made stimulator. The

tissue was mounted parallel with the electrodes. Bipolar, monophasic balance-charged rectangular pulses of 0.8-ms duration and 75 mA current were used.

Kunisawa et al. (1985) performed a pharmacological study of alpha adrenergic receptor subtypes in smooth muscle of **human** urinary bladder base and prostatic urethra.

Thornbury et al. (1992) reported on the mediation of nitric oxide of neurogenic relaxation of the urinary bladder neck muscle in **sheep**. Urinary bladders of sheep of either sex were obtained approximately 15 min after slaughter. Circularly oriented rings were cut from the region of the bladder just above the trigone. These were opened and the mucosa removed by sharp dissection to give strips with approximate dimensions of $10 \times 4 \times 4$ mm. The strips were mounted in organ baths and perfused with Krebs solution. Tension was measured with isometric transducers after field stimulation via platinum ring electrodes.

Hills et al. (1984) used isolated strips of the bladder from female **pigs**. Bladder neck strips were cut longitudinally and horizontally from the region of the bladder just below the trigone. The preparations were stripped of mucosa and trimmed to give a muscle strip of about 2×15 mm.

Klarskov (1987) studied the non-cholinergic, non-adrenergic inhibitory nerve responses of bladder outlet smooth muscle from female Danish Landrace pigs *in vitro*. Trigone strips were taken in an oblique direction from the internal urethral orifice and medially to one of the ureteric orifices, bladder neck strips transversal from the posterior half of the borderline between bladder and urethra, and urethral strips longitudinal from the proximal posterior part.

Teramoto et al. (1997) examined the membrane potential in the proximal urethra of pigs by use of the microelectrode technique.

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C.4.2.3 Effects on external urethral sphincter

PURPOSE AND RATIONALE

In contrast to the smooth musculature of the internal urethral sphincter, the external urethral sphincter is a striate muscle and a part of the urogenital diaphragm (Kuru 1965). Judged from electromyographic studies it is generally accepted that the preponderance of continence depends on the external urethral sphincter. The external urethral sphincter shows a steady tonic discharge under resting conditions. As the bladder fills, there is initially an increase in this activity. When the rise in tension in the bladder wall leads to reflex contraction, the activity in the external urethra sphincter ceases and in remains quiescent during voiding. Parlani et al. (1992) used the external urethral sphincter of the rat as an *in vitro* model to evaluate the activity of drugs on the smooth and striated components of the urinary bladder outlet.

PROCEDURE

Male Wistar rats weighing 360–400 g are sacrificed by decapitation and exsanguination. Through a midline incision of the lower abdomen, the external urethral sphincter (Watanabe and Yamamoto 1979) is isolated from the perineal muscles and surrounding connective tissue and removed in toto. The preparation is placed in oxygenated Krebs solution, and a ring is taken from its middle region. In this area the urethra is encircled by bundles of striated muscle fibers partly interlaced with urethral smooth muscle. The rings are cut to obtain strips that are suspended in a 5-ml organ bath containing Krebs solution at 37 °C. A mixture of 96% O_2 and 4% CO_2 is bubbled into the organ bath. The preparations are connected by means of a silk thread to an isometric strain gauge under a constant load of 1 g. The contractile activity is recorded on a polygraph. Field stimulation is carried out by means of two platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a Grass S11 stimulator. The preparations are allowed to equilibrate for at least 60 min. Square wave pulses are delivered at an intensity between 10 and 60 V, a frequency between 0.1 and 3 Hz, and a duration between 0.1 and 1 s; trains are of 5 s every 5 min. After three consecutive reproducible responses are obtained, drugs are added to the organ bath. The effect of drugs is expressed as the percent of inhibition of contractile response before exposure to drugs and is evaluated as soon as the maximum effect is reached.

EVALUATION

Means ±standard error of the mean are calculated. Statistical evaluation is performed by using Student's *t*-test for paired or unpaired data.

MODIFICATIONS OF THE METHOD

In some studies, denervation of the external urethral sphincter was performed (Somma et al. 1989; Parlani et al. 1992). Rats were anesthetized with 30 mg/kg penthotal i.p., then the major pelvic ganglia, known to provide both sympathetic and parasympathetic innervation to the urinary bladder and the external urethral sphincter (Hulsebosh and Goggeshall 1982; Purinton et al. 1973; Watanabe and Yamamoto 1979) are isolated and bilaterally removed through a small incision of the lower abdomen.

In the same preparations, somatic denervation of the external urethral sphincter was obtained by cutting the pudendal nerves. The paravertebral muscles were carefully dissected through an incision of the skin to exteriorize the sacral plexus. The pudendal nerves were isolated, and 2–3 mm of the nerve were removed. An absorbable sponge soaked with amikain solution was left in place to prevent bleeding and infection. The muscles and the skin were sutured with cat gut. The rats were allowed to recover for 10 to 15 days in individual cages with free access to water and food.

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C.4.2.4 Propagation of impulses in the guinea pig ureter

PURPOSE AND RATIONALE

The mammalian ureter provides one of the most clear examples of electromechanical coupling in smooth muscle. The ureter smooth muscles are electrically and mechanically quiescent but, when depolarized to threshold, they fire an action potential characterized by an unusually long-lasting potential and generate a phasic contraction (Shuba 1977; Brading et al. 1983; Meini et al. 1995). Both the action potential and the accom-

panying contraction critically depend upon the influx of extracellular calcium through voltage-sensitive L-type channels, which are enhanced and blocked by dihydropyridine drugs, Bay K 8644 and nifedipine, respectively. The model predicts that suppression of action potentials at any site of the ureter will suppress the propagation of contraction and peristalsis. (Weiss 1992).

PROCEDURE

Male albino guinea pigs weighing 25–300 g are stunned and bled. The whole kidney and ureter are excised and placed in a Petri dish containing oxygenated Krebs solution for dissection. A 4–5 cm long piece of ureter is dissected from the inferior renal pole and placed in a three-compartment organ bath which enables a separate superfusion of different parts of the organs. Two Perspex partitions are used to separate the renal-, middle- and bladder-sites. They include a window covered with condom rubber: a small hole (about 300 µm) is made in the rubber to enable the passage of the ureter. Proximal to each partition, the renal and bladder ends are pinned to a Sylgard support. The distal portions of the renal and bladder ends are connected via a pulley to isotonic transducers (Basile 7006, load 2 mN) for recording of mechanical activity on a two-channel polygraph. Each compartment is perfused by means of a peristaltic pump at a rate of 1 ml/min with oxygenated Krebs solution at 34 °C.

Electrical field stimulation is applied to either compartment by means of two wire platinum electrodes positioned in parallel with the two sides of the ureter. Square wave pulses (5–25 ms pulse width, 20 V) are automatically delivered every 100 s by means of a GRASS S88 stimulator.

Drugs are applied by superfusion at the middle site. Amplitudes of contraction are recorded.

EVALUATION

Values are calculated as mean ±SEM. Statistical analysis is performed by means of Student's *t*-test or by means of analysis of variance.

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Chapter D
Respiratory activity

D.1 *In vitro* tests

D.1.0.1 Histamine (H_1) receptor binding

PURPOSE AND RATIONALE

Histamine is considered to play a major role in asthmatic attacks. H_1-antagonists have been used since decades as therapeutic agents. This assay is used to determine the affinity of test compounds to the histamine H_1 receptor by measuring their inhibitory activities on the binding of the H_1 antagonist ^{3}H-pyrilamine to a plasma membrane preparation from guinea pig brain.

PROCEDURE

Brains from guinea-pigs are homogenized in ice-cold Tris buffer (pH 7.5) in a Potter homogenizer (1 g brain in 30 ml buffer). The homogenate is centrifuged at 4 °C for 10 min at 50 000 *g*. The supernatant is discarded, the pellet resuspended in buffer, centrifuged as before, and the final pellets resuspended in Tris buffer (1 g fresh weight/5 ml). Aliquots of 1 ml are frozen at –70 °C.

In the competition experiment, 50 µl ^{3}H-pyrilamine (one constant concentration of 2×10^{-9} M), 50 µl test compound (>10 concentrations, 10^{-5}–10^{-10} M) and 100 µl membrane suspension from guinea pig whole brain (approx. 10 mg wet weight/ml) per sample are incubated in a shaking bath at 25 °C for 30 min. Incubation buffer: 50 mM Tris-HCl buffer, pH 7.5.

Saturation experiments are performed with 11 concentrations of ^{3}H-pyrilamine ($0.1–50 \times 10^{-9}$ M). Total binding is determined in the presence of incubation

buffer, non-specific binding is determined in the presence of mepyramine or doxepin (10^{-5} M).

The reaction is stopped by rapid vacuum filtration through glass fibre filters. Thereby the membrane-bound is separated from the free radioactivity. The retained membrane-bound radioactivity on the filter is measured after addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

EVALUATION OF RESULTS

The following parameters are calculated:

- total binding of ^{3}H-pyrilamine
- non-specific binding: binding of ^{3}H-pyrilamine in the presence of mepyramine or doxepin
- specific binding = total binding – non-specific binding
- % inhibition of ^{3}H-pyrilamine binding: 100 – specific binding as percentage of control value

The dissociation constant (K_i) and the IC_{50} value of the test drug are determined from the competition experiment of ^{3}H-pyrilamine versus non-labeled drug by a computer-supported analysis of the binding data.

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D.2
Effects on air ways

D.2.1
Tests in isolated organs

D.2.1.1
Spasmolytic activity in isolated guinea pig lung strips

PURPOSE AND RATIONALE

Several autacoids such as histamine and leukotrienes, induce bronchoconstriction. Histamine is an important mediator of immediate allergic and inflammatory reactions. It causes bronchoconstriction by activating H_1-receptors. Calcium ionophores induce the release of leukotrienes via the 5-lipoxygenase pathway. Leukotrienes are powerful bronchoconstrictors that appear to act on smooth muscles via specific receptors. In this method, drugs are tested for their capability of inhibiting bronchospasm induced by histamine or calcium ionophore. It is used to detect H_1- and leukotriene receptor blocking properties of test compounds.

PROCEDURE

Albino guinea-pigs of either sex weighing 300–450 g are sacrificed with an overdose of ether. The chest cavity is opened and the lungs are removed. They are cut into strips of 5 cm and placed into a physiological saline solution. Thereafter, the lung strips are mounted in an organ bath containing a nutritive solution. The bath is bubbled with carbogen and maintained at 37 °C. Under a pre-load of 0.5 g–3 g, the tissue is left to equilibrate for 30–60 min. Prior to testing, carbachol is added to the bath to test the lung strips' ability of contraction. Twenty min later, two prevalues are obtained by adding the spasmogen

- histamine dihydrochloride 10^{-6} g/ml for 5 min, or
- Ca – ionophore 5×10^{-6} g/ml for 5 min, or
- Leukotriene LTC_4 10^{-9}–10^{-8} g/ml for 10 min, or
- Leukotriene LTD_4 10^{-9}–10^{-8} g/ml for 10 min

to the bath and recording the contractile force at its maximal level. Following a 20 min equilibration period, the spasmogen is administered again. Five minutes thereafter, the test compound is added in cumulative doses from 10^{-8} to 10^{-4} g/ml at 5 or 10 min intervals. The contractile response is determined isometrically.

Test modification:
Inhibition of prostaglandin synthesis

This procedure is identical to the test described above with the exception that the prostaglandin synthesis is inhibited by addition of indomethacin at 10^{-6} g/ml prior to spasmogen administration.

EVALUATION

The percent inhibition of spasmogen induced contraction is calculated.

MODIFICATIONS OF THE METHOD

Lung parenchyma strips from various species were used to measure bronchoactivity by Kleinstiver and Eyre (1979).

A descriptive model of the events occurring during an inflation-deflation cycle using excised rat lungs was proposed by Frazer et al. (1985).

Barrow (1986) measured volume-pressure cycles in air-filled or liquid-filled rabbit lungs ranging from intact lungs with the rib cage immobilized to isolated lungs.

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D.2.1.2 Spasmolytic activity in isolated trachea

PURPOSE AND RATIONALE

The isolated tracheal chain of guinea pigs can be used to test for β-blocking activity (see Sect. A.1.2.7). In addition, this model can be used to test compounds which inhibit bronchospasms. It is used to detect β-sympathomimetic, H_1-receptor blocking and leuko-triene receptor blocking properties of test drugs.

Carbachol is a cholinergic agonist that produces contraction of bronchial smooth muscle by muscarinic stimulation.

Histamine is an important mediator of immediate allergic (type 1) and inflammatory reactions. It causes bronchoconstriction by activating H_1-receptors.

Calcium ionophores induce the release of leukotrienes via the 5-lipoxygenase pathway. Leukotrienes are powerful bronchoconstrictors that appear to act on smooth muscle via specific receptors.

To asses a compound's ability to inhibit carbachol induced bronchospasm via β-receptor activation, a β-receptor blocking agent (for example propranolol) must be added. If relaxation of bronchial smooth muscle is brought about by β-receptor activation, the spasmolytic effect will decrease following propranolol administration.

The effect of bradykinin can be abolished by bradykinin antagonists (Hock et al. 1991).

The effects of potassium channel openers can also be studied in this test (Englert et al. 1992).

PROCEDURE

Albino guinea pigs of either sex weighing 300–550 g are sacrificed by CO_2 narcosis. The entire trachea is dissected out and cut into individual rings (2–3 cartilaginous rings wide). Twelve–fifeteen rings are tied together with silk threads and mounted in the organ bath containing Krebs-Henseleit solution. The tissue is maintained at 37 °C under a tension of 0.5 g and gassed with carbogen. Isometric contractions are recorded via a strain-gauge transduced on a polygraph. Forty-five minutes are allowed for equilibration before the addition of the spasmogen.

The following spasmogens are used:

- carbachol (2×10^{-7} g/ml)
- histamine (10^{-7} g/ml)
- Ca – ionophore for release of leukotrienes
- leukotriene LTC_4 (10^{-9}–10^{-8} g/ml)
- leukotriene LTD_4 (10^{-9}–10^{-8} g/ml)

When the contraction has reached its maximum (initial spasm) after 10–12 min, the standard drug, e.g., isoprenaline (1 ng/ml) or aminophylline (10 ng/ml) is administered. The bronchial responses are allowed to plateau and are recorded. The tissue is rinsed thoroughly and control contractions are induced again by adding spasmogen. After obtaining the initial spasm again, the test drug is added and the contractile force is recorded at its maximal level.

Determination of mechanism of action (testing for β-sympathomimetic effect). After obtaining the initial carbachol induced spasm, propranolol is administered 5 min before the addition of the test drug. Three minutes later, the tissue is challenged by carbachol administration.

EVALUATION

The percent inhibition of carbachol or other spasmogen induced contractions is calculated. From dose-response curves ED_{50} values can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The isolated guinea pig trachea has been proven to be an useful tool for several purposes, e.g., screening procedures and studies on mode of action, e.g., of potassium channel openers.

MODIFICATIONS OF THE METHOD

The molecular mechanisms of β-adrenergic relaxation of airway smooth muscle were described by Kotlikoff and Kamm (1996).

Farmer et al. (1986) studied the effects of epithelium removal on the sensitivity of **guinea-pig** isolated trachealis to bronchodilator drugs.

Wilkens et al. (1992) described a bioassay system for a tracheal smooth muscle-constricting factor using

an isolated guinea pig trachea which was cannulated from both sides, mounted in an organ bath and monitored by a TV camera attached to a microscope. The picture was digitized continuously, and the diameter of the trachea calculated and displayed on a monitor throughout the experiment.

Coleman and Nials (1989) described a versatile, eight-chamber superfusion system for the evaluation of spasmogenic and spasmolytic agents using guinea pig isolated tracheal smooth muscle.

Goldie et al. (1986a) studied the influence of the epithelium on responsiveness of guinea pig isolated trachea to contractile and relaxant agonists.

Lee et al. (1997) studied the effects of bupivacaine and its isomers on guinea pig tracheal smooth muscle. The trachea from the larynx to the carina was removed and cut into single rings. Seven tracheal rings were tied together with the circular muscle running on the same side of the chain, placed into an organ bath containing Krebs Ringer's solution and connected to a force placement transducer for measurement of isometric tension.

Wong et al. (1997) tested the effects of tyrosine kinase inhibitors on antigen challenge of guinea pig lung *in vitro*. Guinea pigs were passively sensitized by a single i.p. injection of 1 mg/kg rabbit IgG antibody against ovalbumin. Bronchial rings, 3 mm in length, were obtained from the hilar bronchi of sacrificed animals and suspended isometrically in an organ bath in Krebs bicarbonate buffer with a resting load of 2 g. To determine maximum antigen-induced contractions, bronchial rings were exposed to increasing concentrations of ovalbumin. To evaluate the role of protein tyrosine kinase in mediating smooth muscle anaphylactic contraction, protein tyrosine kinase inhibitors were preincubated with bronchial rings 30 min before addition of ovalbumin.

Eltze and Galvan (1994) compared the inhibition of preganglionic and postganglionic contraction of the **rabbit** isolated bronchus/trachea by antagonists with selectivity for different muscarinic receptor subtypes with their affinities at M_1, M_2, M_3, and M_4 receptors (Barnes 1993).

For **experiments with vagus nerve stimulation**, the vagi were isolated with rings of the proximal main stem bronchi and a small portion of the distal trachea. The tissues were hung on stainless steel hooks, which passed through the lumen, and were placed in a water-jacketed organ bath filled with Krebs solution plus 2×10^{-5} M choline chloride to promote resynthesis of acetylcholine, 10^{-5} M indomethacin to prevent generation of cyclo-oxygenase products, and 10^{-6} M DL-propranolol to block possible β-adrenoceptor-mediated effects. The preparation was fixed under a resting tension of 1 g for isometric contraction measurement using a force transducer. The vagi were passed around bipolar platinum electrodes held at the surface of the bath. Electrical stimulation was performed with trains (20 Hz, 0.3 ms at 20 V) elicited every 20 min.

For experiments with **field stimulation**, two-ring preparations from the distal trachea of the rabbit were suspended in organ bath in Krebs solution with the above mentioned additions under a resting tension of 1 g. Isometric contractions were elicited at 1 h intervals by continuous electrical field stimulation via platinum electrodes (20 Hz, 0.3 ms at 20 V) for an average 3–7 min to reach a stable plateau.

Vaali et al. (1996) studied in isolated tracheal rings of **rats** and guinea pigs the bronchorelaxing effects of nitric oxide donors. The epithelium of some rings was removed by gentle rubbing. Rat mesenteric rings were cut from the same animals as the bronchi and similarly prepared by removing the endothelium by gently rubbing of the intimate surface.

Farmer et al. (1994) used the isolated trachea from male **ferrets** weighing 1.5–2.5 kg to study the effects of bradykinin receptor agonists.

Toews et al. (1997) assessed the effects of the phospholipid mediator lysophosphatidic acid on the contractile responsiveness of isolated tracheal rings from **rabbits** and **cats**.

Tamaoki et al. (1993) used isolated rings of segmental bronchi from *dogs* to study atypical β-adrenoceptor-(β_3-adrenoceptor)mediated relaxation.

The preparation of **bovine** tracheal smooth muscle for measuring airway responsiveness *in vitro* was described by Hashjin et al. (1995).

Lulich and Paterson (1980) used **human** isolated bronchial muscle preparations and compared the effects of histamine and other drugs with the effects observed on the central and peripheral airways of the rat.

Goldie et al. (1986b) measured the responses of human bronchial strip preparations to contractile and relaxant agonists in preparations from non-diseased and from asthmatic lung obtained 3–15 h post mortem.

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D.2.1.3 Reactivity of the isolated perfused trachea

PURPOSE AND RATIONALE

The mechanism by which the epithelium affects the reactivity of tracheal musculature can be studied using the isolated perfused trachea preparation. Contractile agonists can be added either to the serosal (extraluminal) or to the mucosal (intraluminal) surface (Fedan and Frazer 1992).

PROCEDURE

A 4-cm segment of the trachea of male guinea pigs is removed after sacrifice of the animal and placed for cleaning in modified Krebs-Henseleit solution at 37 °C containing (millimolar) NaCl 113.0, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, and glucose 5.7 (pH 7.4) being gassed with 95% O_2/5% CO_2. The trachea is then attached to a stainless-steel perfusion holder (Munakata et al. 1988), extended to its *in situ* length and placed in an organ chamber (the serosal compartment) at 37 °C containing 25 ml of gassed modified Krebs-Henseleit solution. This solution is also pumped at a constant rate of 30 ml/min through the lumen (mucosal compartment). Responses of the tracheal musculature are obtained by measuring changes in inlet-outlet ΔP between the side holes of indwelling catheters, while the trachea as perfused at a constant rate of modified Krebs-Henseleit solution. The inlet and outlet catheters are connected to the positive and negative sides, respectively, of a differential pressure transducer.

Agonists are added in step-wise increasing, cumulative concentrations. Two consecutive dose-response cur-ves are obtained after the addition either to the serosal or mucosal compartment. The second dose-response curve is obtained 1.5 h after the end of the first, the preparation being washed every 15 min during the intervening period.

EVALUATION

Responses are quantified as ΔP in centimeters of H_2O. Geometric EC_{50} values are determined from least-square analysis of a logit model and are presented along with 95% confidence intervals.

MODIFICATIONS OF THE METHOD

Baersch and Frölich (1996) measured continuously the changes of the diameter of isolated guinea pig tracheal tubes by a newly developed imaging bioassay system. The tracheal tube between larynx and bifurcation was prepared to a length of 1.8–2.5 cm, cannulated at both ends and mounted in a 15 ml organ bath filled and perfused with oxygenated Krebs solution at 37 °C. The lumen of the trachea was perfused with warm (37 °C) Krebs solution from a reservoir that was isolated from the tissue bath. Changes in diameter of the trachea were assessed by computerized video microscopy. The tracheal diameter was used as marker for airway size, thus allowing calculation of muscle contractions under experimental conditions. Following a 30-min equilibrium period, a contraction was induced by intraluminal application of methacholine (0.1 mmol/l) as reference contraction. After washout and return to a stable baseline, cumulative concentration-response curves were obtained by luminal or extraluminal drug application.

Yang et al. (1991) studied the role of epithelium in airway smooth muscle responses to relaxant agents. The results suggested that the epithelium is a relatively weak barrier for lipophilic agents but has a major role as a diffusion barrier to hydrophilic substances.

Munataka et al. (1988, 1989) developed an *in vitro* system to assess the role of epithelium in regulating airway tone using the intact **guinea pig** trachea. The responses to histamine, acetylcholine and hypertonic KCl when stimulated from the epithelial or serosal site were first examined in tracheae with intact epithelium. Then the responses to these agonists were registered after epithelial denudation.

Pavlovic et al. (1989) studied the role of airway epithelium in the modulation of bronchomotor tone in the isolated trachea of **rats**. An organ bath was constructed that permitted independent circulation of fluid within the lumen or around the exterior of the tracheal segment. In one-half of the preparations the epithelium was mechanically removed.

Fernandes et al. (1989) described a co-axial bioassay system, whereby rat cross-cut aorta strip preparations were set up in co-axial assemblies under 500 mg resting tension within a guinea pig tracheal segment serving as donor of the smooth muscle relaxant factor from guinea-pig tracheal epithelium.

Lewis and Broadley (1995) investigated the influence of spasmogen inhalation by guinea pigs upon subsequent demonstration of ovalbumin-induced hyperreactivity in isolated airway tissues. Guinea pigs were sensitized with ovalbumin (i.p.) 14 days before use. *In vitro* airway hyperreactivity induced by ovalbumin inhalation was determined by challenging with aerosolized spasmogen (5-HT, methacholine, the thromboxane-mimetic U-46619, or adenosine) 24 h before and again 18–24 h after the ovalbumin inhalation. One h later, the animals were sacrificed and isolated airways perfused lung halves and tracheal spirals were set up for determination of tissue sensitivity to carbachol, histamine, and adenosine.

Sparrow and Mitchell (1991) used bronchial segments obtained from the lungs of Large-white-/Landrace-cross **pigs** to study the modulation by the epithelium of the extent of bronchial narrowing produced by substances perfused through the lumen.

Mitchell et al. (1989) compared the reactions of perfused bronchial segments and bronchial strips of *pigs* to histamine and carbachol.

Hulsman et al. (1992) recommended the perfused **human** bronchiolar tube as a suitable model.

Omari et al. (1993) studied the responsiveness of human isolated bronchial segments and its relationship to epithelial loss.

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D.2.1.4 Bronchial perfusion of isolated lung

PURPOSE AND RATIONALE

Bronchial perfusion of the isolated lung was described by Sollmann and von Oettingen (1928) as a simple method for studying pharmacological reactions of bronchiolar muscle. The method consists in perfusing fluid down the trachea through the bronchi, and allowing it to escape from the alveoli through scratches on the surface of the lungs. Bronchoconstriction results in a reduced rate of flow, bronchodilatation is indicated by an increased flow. The method has been used to evaluate sympathomimetic drugs by Tainter et al. (1934) and by Luduena et al. (1957).

PROCEDURE

Guinea pigs weighing about 200 g are sacrificed by a head blow. The chest is opened, the trachea cut at the upper end and removed with the lung. The trachea is attached to the cannula of an perfusion apparatus. Only one lung is perfused, the other being tied off. The lower part of the lower lobe is cut off and the rest of the lung surface is scratched deeply assuring maximal pre-medication flow.

The perfusion fluid has the following composition in percentage of anhydrous salts: NaCl 0.659, $NaHCO_3$ 0.252, KCl 0.046, $CaCl_2$ 0.005, $MgCl_2$ 0.0135, NaH_2PO_4 0.01, Na_2HPO_4 0.008, glucose 5%, pH 8.0. The temperature of the perfusion medium is 37.5 °C and the lung is enclosed in a glass cylinder to be protected from variations in the environmental temperature.

The trachea is attached to the cannula of a perfusion apparatus which pumps the solution at a constant rate into a manometric tube connected with the perfused organ. Resistance to the flow (bronchoconstriction) results in an increase in the height of the column of fluid in the manometer. The intensity of bronchodilator effect is measured by the fall of the column in the manometer.

After the lung is attached to a T-shaped cannula, the pump is set in motion and the fluid, after filling the lung, flows out of the system through the third opening of the cannula. By gentle pressure air bubbles are forced out of the lung into the overflow. The lung is then treated in the aforesaid manner, and the upper outlet of the cannula closed. Histamine HCl is added in a concentration of 1 : 2 500 000 as soon as the perfusion starts and the flow is adjusted to obtain a constant progressive increase in pressure.

The drugs are injected near the cannula when the perfusion pressure reaches a level of 500–650 ml of water. The volume injected is always 0.1 ml.

Each drug is tested for bronchodilating activity against the bronchoconstriction induced by histamine in parallel with l-arterenol following a Latin square, including three doses of each drug and three doses of l-arterenol graded at 0.5 log intervals.

EVALUATION

Activity ratios of bronchodilating agents versus the standard can be calculated with a 3 + 3 point assay including confidence limits.

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D.2.1.5 Vascular and airway responses in the isolated lung

PURPOSE AND RATIONALE

The isolated perfused rat lung allows the simultaneous registration of pulmonary vascular and airway responses to various drugs.

PROCEDURE

Male Sprague-Dawley rats weighing 300–350 g are intraperitoneally anesthetized with pentobarbital sodium (50 mg/kg). The trachea is cannulated with a short section of polyethylene tubing, connected to a rodent ventilator, and ventilated with room air enriched with 95% O_2/5% CO_2, with a tidal volume of 4–5 ml/kg and 2 cm H_2O positive end-respiratory pressure. The rats are heparinized with 1 000 units of intravenous heparin and are rapidly exsanguinated by withdrawing blood from the carotid artery.

The lung is exposed by median sternotomy, and a ligature is placed around the aorta to prevent systemic loss of blood. The main pulmonary artery is catheterized, and the lung is removed en block and suspended in a warmed (39 °C), humidified (100%) water-jacketed chamber. An external heat exchanger is used to maintain the temperature of the perfusate and the isolated lung chamber constant throughout the experiment. The perfusate solution (15 ml of heparinized blood and 5 ml modified Krebs-Henseleit solution) is placed in a reservoir and mixed constantly by a mag-

netic stirrer. The lungs are perfused with a peristaltic roller pump at a flow rate of 8–14 ml/min to maintain a physiological baseline pulmonary arterial perfusion pressure of 15 ±0.5 mm Hg. Pulmonary arterial perfusion pressure, airway pressure, and reservoir blood level are continuously monitored, electronically averaged and recorded with a polygraph.

EVALUATION

Changes (increase or decrease) in pulmonary arterial pressure and in airway pressure after injection of test compounds are measured in mm Hg and compared with baseline values.

MODIFICATIONS OF THE METHOD

Bernard et al. (1997) described an isolated perfused lung model with real time data collection and analysis of lung function. Male Sprague Dawley rats were anesthetized with 130 mg/kg pentobarbital i.p. The trachea was cannulated and then ventilated with 5% CO_2 and 95% air at e rate of 60 breath/min and a tidal volume of 2.5 ml. An injection of 650–700 units/kg of heparin was made into the right ventricle. A cannula was placed into the main pulmonary artery. The left ventricle was incised and the lungs were washed free of blood with warmed Krebs-Henseleit bicarbonate buffer with 4.5% BSA and 0.1% glucose. The left atrium was then cannulated to allow outflow of the perfusate. The lung was then removed and suspended in a chamber for perfusion. The flow rate of the perfusate was adjusted to 8–10 ml/min/kg. Ventilation was maintained at 60 breaths/min with humidified and warmed gas. The lung was allowed to recover for 15 min at which time the lung mechanic parameters of flow, volume, transpulmonary pressure, pulmonary artery pressure, weight, resistance, elastance, and positive enddiastolic pressure were measured.

Hauge (1968) studied the conditions governing the pressor responses to ventilation hypoxia in isolated perfused rat lungs.

Uhlig and Heiny (1995) measured the weight of the isolated perfused rat lung during negative pressure ventilation for quantitating edema formation in the isolated lung.

Uhlig and Wollin (1994) described an improved setup for the isolated perfused rat lung. Breathing mechanic, such as tidal volume, pulmonary compliance, and pulmonary resistance, as well as perfusate characteristics, such as pulmonary vascular resistance, pulmonary pre- and postcapillary resistance, perfusate pH, P_{O2}, and P_{CO2}, and the capillary filtration coefficient were determined.

Byron et al. (1986) used the isolated perfused rat lung preparation for the study of aerosolized drug deposition and absorption.

Hendriks et al. (1999) published a modified technique of isolated left lung perfusion in the rat.

Riley et al. (1981) determined the tissue elastic properties of saline-filled isolated hamster lungs by measuring the pressure-volume relationships and studied the prevention of bleomycin-induced pulmonary fibrosis by *cis*-4-hydroxy-L-proline.

Lewis and Broadley (1995) tested the influence of spasmogen inhalation by **guinea pigs** upon subsequent demonstration of albumin-induced hyperreactivity in isolated airway tissues, such as perfused lung halves and tracheal spirals.

Anglade et al. (1998) measured the pulmonary capillary filtration coefficient in isolated **rabbit** lungs which were suspended by a string tied around the tracheal cannula from a counter-balanced force transducer to perform continuous weight measurement. To measure the pulmonary capillary filtration coefficient, pulmonary venous pressure was raised stepwise which results in an initial large weight gain for a few seconds followed by a slower rate of weight gain. The pulmonary capillary filtration coefficient was calculated on the slow phase of the weight curve by a time zero extrapolation or from the slope of the curve.

Nakamura et al. (1987) studied neurogenic pulmonary edema in lung perfusion preparations *in situ* in the **dog**.

Allen et al. (1993) studied the cardiovascular effects of a continuous prostacycline administration into an isolated *in situ* lung preparation in the dog.

Pogrebniak et al. (1994) investigated the influence of tumor necrosis factor in an isolated lung perfusion model in **pigs**.

The fluid filtration coefficient before and after infusion of *Escherichia coli* endotoxin was measured in excised **goat** lungs by Winn et al. (1988).

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D.2.2 *In vivo* tests

D.2.2.1 Bronchospasmolytic activity in anesthetized guinea pigs (Konzett-Rössler method)

PURPOSE AND RATIONALE

The principle war first described by Kiese (1935). Konzett and Rössler (1940) published a method suitable for screening procedures which found worldwide acceptance. A survey on the history and further modifications was given by Döring and Dehnert (1997).

The method is based on registration of air volume changes of a living animal in a closed system consisting of the respiration pump, of the trachea and the bronchi as well as of a reservoir permitting measurement of volume or pressure of excess air. Bronchospasm decreases the volume of inspired air and increases the volume of excess air. Thus, the degree of bronchospasm can be quantified by recording the volume of excess air. Administration of spasmogens like acetylcholine, histamine, bradykinin, serotonin, ovalbumin, PAF, substance P, methacholine or leukotrienes, results in contraction of bronchial smooth muscle.

The method permits the evaluation of a drug's bronchospasmolytic effect by measuring the volume of air, which is not taken up by the lungs after bronchospasm.

PROCEDURE

Guinea-pigs of either sex weighing 250–500 g are anaesthetized with 1.25 g/kg i.p. urethane. Pentobarbital (60 mg/kg s.c.) and alcuronium chloride (1 mg/kg s.c.) are to be preferred when the bronchospasm is elicited by PAF or substance P. Anesthesia has to be deep enough in order to prevent influence of spontaneous respiration. The trachea is cannulated by means of a two way cannula, one arm of which is connected to the respiratory pump and the other to a Statham P23 Db transducer. The animal is artificially respired using a Starling pump with an inspiratory pressure set at 90–120 mm of water, an adequate tidal volume of 3 ml/100 g body weight and a frequency of 60 strokes per minute. Excess air, not taken up by the lungs, is measured and recorded on a polygraph. The internal jugular vein is cannulated for the administration of spasmogens and test compounds. The carotid artery is cannulated for measuring blood pressure.

Testing

Guinea-pigs receive the following spasmogens by i.v. administration:

- acetylcholine hydrochloride (20–40 µg/kg), or
- methacholine (20–40 µg/kg), or
- histamine dihydrochloride (5–20 µg/kg), or
- bradykinin triacetate (10–20 µg/kg), or
- ovalbumin (1 mg/kg), or
- PAF (25–50 ng/ml), or
- leukotrienes LTC_4, LTD_4 (about 1 µg/kg), or
- substance P (0.5 µg/kg).

After obtaining two bronchospasms of equal intensity, test compounds are administered i.v., p.o., s.c. or intraduodenally.

The spasmogen is given again at the following time intervals:

- 5, 15 and 30 min after i.v. administration of the drug
- 15, 30 and 60 min after intraduodenal administration of the drug
- 30 and 60 (sometimes also 120) min after p.o. administration of the drug.

The following standard compounds are used:

- atropine sulfate (0.01 mg/kg, i.v.) to inhibit acetylcholine or methacholine induced spasms
- aminophylline (6 mg/kg, i.v.) to inhibit bradykinin induced spasms
- tolpropamine-HCl (0.2 mg/kg) to inhibit histamine induced spasms
- imipramine-HCl (3–5 mg/kg) to inhibit serotonin-kreatinin-sulphate induced spasms.

EVALUATION

Results are expressed as percent inhibition of induced bronchospasm over the control agonistic responses. The ED_{50} value is calculated.

CRITICAL ASSESSMENT OF THE METHOD

The "Konzett-Roessler"-method has been proven to be a standard procedure in respiratory pharmacology being modified by several authors (Rosenthale and Dervinis 1968).

MODIFICATIONS OF THE METHOD

Forced insufflation was proposed as a simple but accurate inhalation procedure for investigating the activity of anti-asthmatic drugs in guinea pigs by Schiantarelli et al. (1982).

Lundberg et al. (1983), Belvisi et al. (1989), Miura et al. (1994) determined airway opening pressure (P_{ao}) as an index for tracheobronchial resistance to air flow.

Orr and Blair (1969) and Riley et al. (1987) sensitized rats intravenously with a potent antiserum to ovalbumin, obtained by infecting ovalbumin-sensitized donor rats with the parasitic nematode *Nippostrongylus brasiliensis* in order to boost IgE antibody production. The anesthetized animals were challenged with ovalbumin 48 h after sensitization and the subsequent increase in tracheal pressure was recorded.

Collier et al. (1993), Collier and James (1967) published a modification of the Konzett-Rössler-method using the forced re-inflation to overcome the severe bronchoconstriction occurring in sensitized guinea pigs.

Further modifications are by Schliep et al. (1986), Marano and Doria (1993).

Groeben and Brown (1996) measured changes in the cross-sectional area of conducting airways by cumulative doses of ipratropium with and without gallamine, a selective M_2 muscarinic receptor blocker, and after metaproterenol in anesthetized **dogs** using high-resolution computed tomography. Using a Somatom Plus scanner (Siemens), 50 to 55 contiguous scans were obtained, starting approximately 5 mm above the origin of the right upper lobe bronchus from the trachea and proceeding caudally using 1-mm table feed and 2-mm slice thickness. The dogs were anesthetized with thiopental. After paralysis was induced by succinylcholine, the trachea was intubated and the lungs ventilated with a volume-cycled ventilator with 100% oxygen. During the scans, the dogs were apneic at function residual capacity (approximately 2 min). Images were reconstructed using a high-spatial frequency algorithm.

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D.2.2.2 Effect of arachidonic acid or PAF on respiratory function *in vivo*

PURPOSE AND RATIONALE

Based on the classical method of Konzett and Rössler (1940), Lefort and Vargafting (1978), Vargafting et al. (1979) studied the effects of arachidonic acid and PAF on respiratory function of guinea pigs *in vivo*.

Arachidonic acid is metabolized into thromboxane (TXA_2) and prostacyclin (PGI_2). TXA_2 produced in the lung leads to bronchoconstriction, which is independent from circulating platelets and leukotrienes; TXA_2 produced intracellularly in platelets induces a reversible thrombocytopenia. PGI_2 produced in the vessel wall leads to the reduction of systolic and diastolic blood pressure. All three effects are inhibited by drugs which block cyclo-oxygenase. In contrast, agents which block thromboxane synthetase inhibit bronchoconstriction and thrombocytopenia, but lead to a potentiation of blood pressure reduction.

In contrast to arachidonic acid, PAF as inducer leads to bronchoconstriction, which is platelet-dependent. In addition, PAF induces thrombocytopenia, leukocytopenia, reduction of blood pressure and increase of hematocrit. These effects are also reversible, but more persistent than those induced by arachidonic acid, and quickly result in tachyphylaxis. The test allows to evaluate the sites of action of drugs, which interfere with the mechanisms of broncho-constriction and thrombocytopenia; in an *in vivo*-model guinea pigs are challenged with the spasmogens and platelet-aggregating substances arachidonic acid or PAF (platelet activating factor).

PROCEDURE

Male guinea pigs (Pirbright White) weighing 300–600 g are anesthetized with 60 mg/kg pentobarbital sodium (i.p.). One of the jugular veins is cannulated for the administration of spasmogen and test compound. Both external carotid arteries are cannulated; one is connected to a pressure transducer to register blood pressure, the other is used for blood withdrawal. The trachea is connected to a Starling pump with an inspiratory pressure set of 80 mm H_2O, an adequate tidal volume of approx. 10 ml/kg body weight and a frequency of 70–75 strokes/min. Spontaneous respiration is inhibited by intravenous injection of pancuronium (4 mg/kg) or gallamine (2 mg/kg) on time.

In some experiments, pulmonal ß-receptors are blocked by intraperitoneal administration of propranolol (2 mg/kg).

Excess air, not taken up by the lungs, is conducted to a transducer with bronchotimer (Rhema, Germany) which translates changes in air flow to an electrical signal. Changes in air flow and arterial blood pressure are recorded continuously.

Animals receive multiple intravenous injections of the same dose of arachidonic acid (Sigma, 250–600 µg/kg prepared from a stock solution 10 mg/ml ethanol, 1 : 20 dilution with Na_2CO_3) until two bronchospasms of equal intensity are obtained. The test compound is administered intravenously and the spasmogen is given again at the following intervals: 2, 10, 20 and, if necessary, 30 min after administration of the drug.

Ordinarily, the lung has to be passively dilated (bronchotimer) after each of the bronchoconstrictions. Immediately before and 30–45 s after each of the arachidonic acid applications, approx. 50 µl blood are collected into Na-EDTA-coated tubes. The number of thrombocytes is determined with a platelet analyzer (Becton Dickinson Ultra-Flo-100 or Baker 810) in 10 µl samples of whole blood.

PAF (Paf-acether C16, Bachem, 0.03–0.04 µg/kg in 0.9% saline + 0.1% human serum albumin) as inducer is injected intravenously 60 min before, 5 min and, if necessary, 60 min after intravenous drug administration. Blood samples are collected 30 s before and 15 s after each of the PAF applications. The number of leuko-cytes and hematocrit values are determined automatically (TOA-microcell counter CC 108, Colora Meßtechnik).

Standard compounds:

- dazoxiben HCl (inhibitor of thromboxane synthetase, TSI)
- acetylsalicylic acid- (inhibitor of cyclo-oxygenase, COI)

EVALUATION

Percent inhibition or increase of bronchospasm, reduction of blood pressure, thrombocytopenia, leukocytopenia and hematocrit following test drug administration are calculated in comparison to control values before drug treatment. For the reduction of blood pressure, both the magnitude [mm Hg, systolic and diastolic] and the duration [min] are determined. Even a sole increase in duration of blood pressure reduction is considered as an increase of the effect. From the pattern profile of the influence on bronchoconstriction, thrombocytopenia and blood pressure reduction, the mechanism of action of a test drug is concluded:

- inhibitor of thromboxane synthetase
- inhibitor of cyclo-oxygenase
- other effect = no profile

In addition, the inherent action of the test substance on blood pressure is determined before arachidonic acid- or PAF-administration.

MODIFICATIONS OF THE METHODS

Kagoshima et al. (1997) used a modification of the Konzett-Rössler-method to test the suppressive effects of a PAF-antagonist on asthmatic responses in guinea pigs actively sensitized with ovalbumin. The immediate and the late asthmatic response were measured by the oscillation method according to Mead (1960).

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D.2.2.3 Bronchial hyperreactivity

PURPOSE AND RATIONALE

Symptoms like asphyctic convulsions resembling bronchial asthma in patients can be induced by inhalation of histamine or other bronchospasm inducing agents in guinea pigs. The challenging agents are applied as aerosols produced by an ultra-sound nebulizer. The first symptoms are increased breathing frequency, forced inspiration, and finally asphyctic convulsions. The occurrence of these symptoms can be delayed by antagonistic drugs. Pre-convulsion time, i.e. time until asphyctic convulsions, can be measured.

PROCEDURE

Ten male albino guinea pigs weighing 300–400 g per group are used. The inhalation cages consist of 3 boxes each ventilated with an air flow of 1.5 l/min. The animal is placed into box A to which the test drug or the standard is applied using an ultra-sound nebulizer LKB NB108 which provides an aerosol of 0.2 ml solution of the test drug injected by an infusion pump within 1 min. Alternatively, the animal is treated orally or subcutaneously with the test drug or the standard. Box B serves as a sluice through which the animal is passed into box C. There, the guinea pig is exposed to an aerosol of a 0.1% solution of histamine hydrochloride provided by an ultra-sound nebulizer (De Vilbiss, Model 35 A). Time until appearance of asphyctic convulsions is measured. Then, the animal is immediately withdrawn from the inhalation box. The aerosols are removed from the back wall of the boxes by applying low pressure.

EVALUATION

Percent of increase of pre-convulsion time is calculated versus controls. ED_{50} values can be found, i.e. 50% of increase of pre-convulsion time.

CRITICAL ASSESSMENT OF THE METHOD

The "guinea pig asthma" has been applied as useful method in various modifications by many laboratories.

MODIFICATIONS OF THE METHODS

Simple methods to test bronchospasmolytic activity in conscious animals, called "thoracography" were described by Herxheimer (1956) Olsson (1971), Beume et al. (1985). A silicon tube with a diameter of 1 mm is filled with mercury and serves as strain gauge applied as belt around the thorax of conscious guinea pigs. Changes in electrical resistance due to breathing movements of the thorax are registered. The animals are exposed in a Plexiglas chamber to acetylcholine nebulized by an ultrasonic device. Time until onset of coughing indicated by an increase of signal amplitude and of severe asthmatic dyspnoea is registered.

Immunological factors are involved in bronchial hyperreactivity (Reynolds 1991).

Harris et al. (1976) immunized rabbits with thermophilic actinomyces antigen (Micropolyspora faeni). Lesions resembling hypersensitivity in man were found, characterized by a mononuclear cell interstitial reaction and a marked increase in the number of intra-alveolar cells.

Ufkes et al. (1983) induced bronchial and cardiovascular anaphylaxis in Brown-Norway rats, sensitized with trinitrophenyl haptenized ovalbumin and $AlPO_4$ as adjuvant 12 days prior to challenge with trinitrophenyl haptenized bovine serum albumin intravenously.

Raeburn et al. (1992) gave a survey on techniques for drug delivery to the airways and the assessment of lung functions in animal models including parameters such as lung compliance and airway resistance.

Elwood et al. (1992) studied the effects of dexamethasone and cyclosporin A on the airway hyperresponsiveness and the influx of inflammatory cells into bronchoalveolar lavage fluid seen 18 to 24 h after exposure to aerosolized ovalbumin in actively ovalbumin-sensitized Brown-Norway rats.

A model of bronchial hyperreactivity after active anaphylactic shock in conscious guinea pigs has been described by Tarayre et al. (1990). The guinea pigs were sensitized by an intramuscular injection of a large dose of ovalbumin in Freund's adjuvant. The adminis-

tration of ovalbumin to induce anaphylactic shock was by aerosol. Bronchial hyperreactivity to histamine was observed 3–6 h after the anaphylactic shock.

The importance of eosinophil activation for the development of allergen-induced bronchial hyperreactivity was underlined by Santing et al. (1994). A significant increase in bronchoreactivity to histamine was observed at 6 h after allergen exposure, which was associated with an increase of eosinophils in the bronchoalveolar lavage and an increase in the eosinophil peroxidase activity

The increased pulmonary vascular permeability may be related to the adult respiratory distress syndrome in man (Snapper and Christman 1989).

The infusion of small amounts of *Escherichia coli* endotoxin into chronically instrumented awake **sheep** results in well-characterized pulmonary dysfunction (Brigham and Meyrick 1986).

One animal model associated with both increased airway responsiveness and pulmonary inflammation is endotoxemia in sheep (Hutchinson et al. 1983).

The effect of a platelet activating factor receptor antagonist on the sheep's response to endotoxin was studied by Christman et al. (1987).

Chiba and Misawa (1995) characterized muscarinic cholinoceptors in airways of antigen-induced airway hyperresponsive rats. The animals were sensitized with 2,4-dinitrophenylated *Ascaris suum* extract together with *Bordetella pertussis* (2×10^6) as an adjuvant and were boosted with 2,4-dinitrophenylated *Ascaris suum* extract 5 days later. Isometric contractions of the circular muscle of isolated bronchial rings after addition of increasing doses of acetylcholine were measured with a force-displacement transducer.

Laboratory infection of **primates** with *Ascaris suum* may provide a model of allergic bronchoconstriction (Patterson et al. 1983; Pritchard et al. 1983; Eady 1986).

Richards et al. (1986) used various models of airway hypersensitivity, such as inhalation of *Ascaris suum* antigen in monkeys and dogs.

Rylander and Marchat (1988) studied the effect of a corticosteroid on an acute inflammation in the lungs of guinea pigs exposed to an aerosol of bacterial endotoxin. The subsequent inflammatory response was evaluated counting the number of cells obtained from airway lavage and in the lung interstitium as well as the chemotactic effect of alveolar macrophages.

Hatzelmann et al. (1996) reported on automatic leukocyte differentiation in broncho-alveolar lavage fluids of guinea pigs and Brown-Norway rats using an automatic cell analyzing system (Cobas Helios 5Diff; Hoffmann-La Roche; Grenzach-Wyhlen, Germany).

Minshall et al. (1993) demonstrated that neonatal immunization of **rabbits** with *Alternia tenuis* can lead to the development of persistent airway hyperresponsiveness.

Okada et al. (1995) studied late asthmatic reactions in **guinea pigs** sensitized with *Ascaris* antigen. They evaluated interleukin-1 production by immunostaining with anti-IL-1β antibody and elucidated the action of IL-1 in late asthmatic reactions with recombinant IL-1 receptor antagonist.

Folkerts et al. (1995) found that intratracheal inoculation of *parainfluenza type 3 virus* to guinea pigs induces a marked increase of airway responsiveness to increasing doses of intravenous histamine. In spontaneously anesthetized breathing guinea pigs, inhalation of an aerosol containing the nitric oxide precursor L-arginine completely prevented the virus-induced hyperresponsiveness to histamine.

Fryer et al. (1994, 1997) measured M_2 muscarinic receptor function in anesthetized and paralyzed guinea pigs by electrical stimulation of both vagus nerves producing bronchoconstriction (measured as pulmonary inflation pressure) and bradycardia.

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D.2.2.4
Body plethysmography and respiratory parameters after histamine-induced bronchoconstriction in anesthetized guinea pigs

PURPOSE AND RATIONALE

Guinea pigs can be placed in a plethysmograph for measurement of respiratory parameters. Respiratory frequency and respiratory amplitude are recorded. The decrease of respiratory amplitude (diminished respiratory volume due to bronchoconstriction) and the reflectory increase of respiratory frequency after histamine inhalation are attenuated by bronchodilatatory drugs. Additional respiratory parameters can be recorded using a Fleisch tube and a catheter inserted into the pleural cavity (Englert et al. 1992). The method can be used for various purposes, e.g., to evaluate the antagonism against bradykinin-induced bronchoconstriction (Wirth et al. 1991, 1993) or the bronchodilator effects of potassium channel openers (Englert et al. 1992) or to measure the effect of morphine on respiration in rats (Kokka et al. 1965).

PROCEDURE

Guinea pigs of either sex weighing 400–600 g are anesthetized with 70 mg/kg pentobarbital i.p. The trachea, pleural cavity, jugular vein, and carotid artery are prepared and cannulated. The animals are mechanically ventilated with a Starling respiratory pump which delivers an inspiration volume that represents a tracheal pressure of 8 cm water at a rate of 60 strokes/min. Succinylcholine chloride at a dose of 1 mg/kg is given i.v. to prevent interference from spontaneous respiration. The guinea pigs are placed inside a whole body plethysmograph and tracheal, pleural, venous and arterial catheters are connected to onset ports in the wall of the plethysmograph box. The tracheal port is then connected with the respiration pump. **Airflow rate** into and out of the plethysmograph are measured as pressure difference with a No. 000 Fleisch tube and a differential pressure transducer (Fa. Hellige, PM 97 TC). Airflow is calibrated by passing compressed air through a rotameter. The **tidal volume (V_T)** is calculated from the flow signal. **Transpulmonary pressure (P_{TP})** is measured with a differential pressure transducer (Hellige, PM 97 TC), with one side attached to a catheter inserted into the right pleural cavity and the other side connected to a side port of the tracheal cannula. P_{TP} is calibrated with a water manometer. Signals from airflow, tidal volume and transpulmonary pressure are fed into an on-line computer system (PO-NE-MAH®, Model PF-1, Storrs) for calculation of **pulmonary resistance (R_L)** and **dynamic lung compliance (C_{DYN})**.

These parameters are calculated for each breath with a sampling rate of 100 s^{-1} for each circle. Flow and pressure signals for computation are obtained from a PLUGYS measuring system (Fa. Hugo Sachs Elektronik, Freiburg, Germany). Systemic arterial pressure is measured using a Statham pressure transducer (P 23 Db). Heart rate is computed from pressure pulses (Döring and Dehnert 1997).

Three doses of test compound or standard are injected intravenously. Saline injections serve as controls. Intravenous injections of histamine (0.5–2 μg/kg) lead to a short decrease in C_{DYN} and to a short increase in R_L by approximately 200% compared with baseline. Challenges are repeated at 5-min intervals, yielding the same increase in R_L during the whole 1-h experimental period. After 3 reproducible responses the test agent is administered intravenously 1 min before the histamine injection.

To evaluate test compounds for inhalation route, aerosols are generated with an ultrasonic nebulizer (LKB, model NB 108) and are administered to the animals through a shunt in the afferent limb of the respiratory pump, allowing the inspired air to pass through the nebulizer chamber before entering the animals lungs.

EVALUATION

Inhibition of histamine induced bronchoconstriction by various doses of test compound and standard is recorded. ED_{50} values for inhibition in R_L are calculated. Furthermore, the time course of histamine antagonism can be evaluated. Compounds can be tested either after iv. injection of histamine (prevention) or during intravenous infusion of histamine (intervention).

CRITICAL ASSESSMENT OF THE METHOD

Whole body plethysmography has been proven to be a useful tool in respiratory pharmacology for studies on the antagonism against various bronchoconstrictors, such as histamine and bradykinin, as well as for airway pharmacology of potassium channel openers.

MODIFICATIONS OF THE METHOD

Several authors use body plethysmography to study respiratory functions in animals (Amdur and Mead 1958; Blümcke et al. 1967), Pennock et al. 1979; Agarwal (1981), James and Infiesto (1983), Kisagawa et al. (1984), Griffith-Johnson (1988), Danko and Chapman (1988), Ball et al. (1991), Chand et al. (1993).

The effect of β-blockers on pulmonary function and bronchoconstrictor responsiveness in **guinea pigs** and rats has been studied by Chapman et al. (1985).

Finney and Forsberg (1994) developed a technique for quantification of nasal involvement in a guinea pig plethysmograph. Nasal and lower respiratory system conductance could be measured simultaneously in anesthetized animals.

A whole body plethysmograph for conscious animals has been described by Elliott et al. (1991) and improvements by Linton (1991).

Studies of bronchospasmolytic agents with aerosol challenge in conscious guinea pigs using a double chamber plethysmograph box have been reported by Schlegelmilch (1991).

Ball et al. (1991) described a method for the evaluation of bronchoactive agents in the conscious guinea pig. The method involves the use of "head out" whole body plethysmographs from which respiratory rate can be recorded by monitoring respiration-related changes in pressure within the body chamber.

Hey et al. (1995) used a head-out, whole body plethysmograph to examine the effects of $GABA_B$ receptor agonists on minute ventilation, tidal volume and respiratory rate due to room air and carbon dioxide-enriches gas hyperventilation in conscious guinea pigs.

Murphy et al. (1998) developed a method for chronic measurement of pleural pressure in conscious *rats*. Pleural pressures were measured by surgically implanting a fluid-filled polyurethane catheter attached to a pressure-sensitive radiotelemetry transmitter (Model TA11PA-C40, Data Sciences Int., St. Paul, MN) beneath the pleural surface. A compatible receiver (Model RLA0120) and a data acquisition and analysis software system (LabPRO, Version 3) sampling at a rate of 500 Hz were used to analyze the telemetric signals.

Sinnett et al. (1981) described a fast integrated flow plethysmograph for small animals (**mice**).

Simple methods to determine respiratory parameters in small animals were described by Schütz (1960), Höbel et al. (1971).

Schlenker (1984) and Schlenker and Metz (1989) evaluated ventilatatory parameters in dystrophic **Syrian hamsters**.

Wasserman and Griffin (1977) studied bronchoactivity in the intact anesthetized **dog**.

Paré et al. (1976) determined pulmonary resistance and dynamic lung compliance in **rhesus monkeys**.

Wegner et al. (1984) measured dynamic respiratory mechanics in monkeys by forced oscillations generated by a loudspeaker in an airtight chamber.

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D.2.2.5 Pneumotachography in anesthetized guinea pigs

PURPOSE AND RATIONALE

The use of a pneumotachograph based on the principle of the Fleisch-tube and of additional pressure transducers allows simultaneous measurements of several respiratory and circulatory parameters in anesthetized guinea pigs (de la Motta 1991).

PROCEDURE

Guinea pigs (Pirbright white) weighing 300–400 g are anesthetized with 1.5 g/kg urethane i.p. The animals are shaved ventrally at the neck, placed on a heated operating table, and fixed at the upper extremities. A metal cannula with a blunted tip is inserted into the trachea and secured with a loop within the caudal section of the trachea. A thin plastic catheter is inserted into the esophagus and the tip located inside the thorax in order to register intrathoracic pressure. Furthermore, the cephalic vein at one side and the carotid artery on the opposite side are cannulated. The tracheal cannula is connected with pieces of tubing to a Fleisch

tube (pneumotachograph), size 0000. In order to avoid water condensation, the Fleisch tube is heated. The Fleisch tube is connected with a sensitive differential pressure transducer with a range of 2 cm H_2O (Validyne, model MP 45 -xx-871). One side of another pressure differential transducer with a range of 20 ml H_2O is connected with the esophageal catheter, the other side remaining open to room air. Both Validyne pressure transducers are connected to a separate preamplifier. For recording of the arterial blood pressure a Gould pressure transducer, Type P23Gb, is used. The signals for airflow and esophageal pressure are monitored at the output of the preamplifier with a digital 2-channel oscilloscope. To obtain various respiratory and circulatory parameters from the three primary signals they are calculated by certain formulae by an analogue computer (Buxco Pulmonary Mechanics Analyzer, Model 6). The following parameters are presented at the output of the instrument as analogue electrical signals:

Circulation

Systolic blood pressure, diastolic blood pressure, mean blood pressure

Respiration

Tidal volume, respiratory volume per minute, respiratory rate

Pulmonary mechanics

Airway resistance, dynamic compliance, end-respiratory work.

A multi-channel recorder (Graphtec Linearcorder Mark VII) serves a functional check on the Buxco Analyzer and as analogue presentation of the calculated parameters. Data processing is performed by a 12-channel A/D converter (Buxco Data Logger, Model D/C-12 F/V) which digitizes the analogue output signals of the Buxco Analyzer and sends them through a serial interface (RS232) to an IBM PC. A special software program (Lomask 1987; Hastings 1990a,b) provides a flexible facility for data reduction and statistical evaluation.

EVALUATION

For each individual experiment the data of the last 5 min before the first substance application are averaged and used as controls. The response values after substance application are then expressed as percentages of the controls. In this way each animal serves as its own control. For an analysis of the results the response values are averaged over certain time intervals.

CRITICAL ASSESSMENT OF THE METHOD

The method described in great detail by de la Motta (1991) may be modified using different equipment according to individual needs.

MODIFICATIONS OF THE METHOD

Measurement of respiratory parameters is based on earlier studies to be mentioned for historical reasons (Gad 1880; Pflüger 1882; Zwaardemaker and Ouwehand 1904; Jaquet 1908; Rohrer 1915; Gildemeister 1922; Fleisch 1925; v. Neergaard and Wirz 1927; survey by Döring HJ 1991).

Lorino et al. (1988) assessed respiratory mechanics of histamine bronchopulmonary reactivity in guinea pigs.

O'Neil et al. (1981) published a comparative study of respiratory responses to bronchoactive agents in rhesus and cynomolgus monkeys.

Rayburn et al. (1989) described a computer-controlled pulmonary function system for studies in large animals.

Five methods of analyzing respiratory pressure-volume curves have been compared by Lai and Diamond (1986).

A specially designed pneumotachograph that is placed inside the trachea of guinea pigs was described by Santing et al. (1992) allowing the evaluation of airway functions in conscious, unstressed animals.

Lorino et al. (1993) estimated the changes in end-respiratory lung volume accompanied histamine-induced bronchoconstriction in anesthetized, paralyzed, and mechanically ventilated guinea pigs from measurements of thoracic cross-sectional area, assessed from the voltage induced by an external uniform magnetical field in a pickup coil encircling the rib cage.

Gozzard et al. (1996) evaluated the effects of PDE-inhibitors in **New Zealand White rabbits** which were immunized within 24 h of birth with *Alternaria tenuis* antigen. Spontaneously breathing rabbits were intubated in neuroleptanalgesia with a cuffed endotracheal tube connected to a thermoregulated Fleisch pneumotachograph to allow measurement of tidal air flow. An oesophageal balloon catheter was inserted to provide a measure of intra-pleural pressure and transpulmonary pressure. Antigen challenge was performed with inhaled *Alternaria tenuis* extract.

An excellent survey on various methods and equipment to measure air flow is given by Döring and Dehnert (1997).

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D.2.2.6 Airway microvascular leakage

PURPOSE AND RATIONALE

Plasma exudation in guinea-pig airways *in vivo* can be determined by Evans Blue dye and is fairly correlated with radiolabelled albumin (Rogers et al. 1989). This method can be used to study the antagonism against bradykinin- and platelet-activating factor-induced airway microvascular leakage and vagal stimulation-induced airway responses (Sakamoto et al. 1992, 1994).

PROCEDURE

Female Dunkin-Hartley guinea pigs weighing 380–600 g are anesthetized with an initial dose of 1.5 g/kg urethane injected i.p. Additional urethane is given i.v. 30 min later to achieve an appropriate level of anesthesia. A tracheal cannula is inserted into the lumen of the cervical trachea, a polyethylene catheter into the left carotid artery to monitor blood pressure and heart rate and another polyethylene catheter into the external jugular vein for administration of drugs. The animals are connected to a constant volume mechanical ventilator and then given an injection of 1.0–1.5 mg/kg suxamethonium i.v. to prevent interference with spontaneous respiration. A tidal volume of 10 ml/kg and a frequency of 60 strokes/min are used.

Lung resistance is measured as an index of airway function and monitored throughout the experiment. Transpulmonary pressure is measured with a pressure transducer with one side attached to a catheter inserted into the right pleural cavity and the other side attached to the side port of the intratracheal cannula. Airflow is measured by a pneumotachograph connected to a pressure transducer. The signals of the transducers are used for instantaneous calculation of lung resistance by an appropriate computer program.

The test compound (bradykinin receptor antagonist) is given intravenously. Ten min later, Evans Blue dye (20 mg/ml) is injected i.v. for 1 min. After 1 min, bronchoconstriction and microvascular leakage is induced by injection of bradykinin or by inhalation of bradykinin or PAF or vagal stimulation.

Six min after induction of leakage, the thoracic cavity is opened, and a cannula is inserted into the aorta through a ventriculotomy. Perfusion is performed with 100–150 ml 0.9% saline at a pressure of 100–120 mm Hg in order to remove the intravascular dye from the systematic circulation. Blood and perfusion liquid are expelled through an incision in the right and left atrium. Subsequently, the right ventricle is opened, and the

pulmonary circulation is perfused with 30 ml of 0.9% saline. The lungs are then removed, and the connective tissue, vasculature, and parenchyma are gently scraped. The airways are divided into 4 components: lower part of the trachea, main bronchi, the proximal 5 mm portion, and the distal intrapulmonary airways. The tissues are blotted dry, and then weighed. Evans Blue dye is extracted in 2 ml of formamide at 40 °C for 24 h, and measured in a spectrophotometer at 620 nm.

EVALUATION

Evans Blue dye concentration, expressed as ng/mg tissue, as well as lung resistance are compared by statistical means (unpaired Student's *t*-test or Mann-Whitney U test) between treated groups and controls receiving the challenge only.

MODIFICATIONS OF THE METHOD

Boschetto et al. (1989) tested the effect of antiasthma drugs on microvascular leakage in guinea pig airways. Microvascular leakage was induced by intravenous injection of platelet-activating factor (50 ng/kg) which acts directly on venular endothelial cells, and measured by quantifying extravasation of Evans blue dye.

Xu et al. (1998) induced pulmonary edema in rats by injection of 20 µg/kg angiotensin I and studied the suppression by ACE-inhibitors, ATII antagonists and α-adrenergic receptor blockers.

Rapidly developing pulmonary edema was induced by intravenous injection of 1.2 mg/kg of the GABA agonist bicuculline in rats and the role of endogenous endothelin was examined by Herbst et al. (1995).

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D.2.2.7 Isolated larynx *in situ*

PURPOSE AND RATIONALE

The *in situ* isolated larynx of rats has been recommended by Willette et al. (1987) for evaluation peripheral opiate receptor antagonists. Peripheral opioid-induced laryngospasm and central opioid-induced respiratory depression can be measured simultaneously. Fentanyl citrate stimulates both peripheral and central opiate receptors, whereas [D-Ala^2-Met^5]-enkephalinamide stimulates only peripheral opiate receptors. Compounds that inhibit both laryngeal and respiratory effects of fentanyl, e.g., naloxone HCl, can be considered both central and peripheral opiate antagonists. Compounds that inhibit only the peripheral effects of fentanyl, e.g., naltrexone methylbromide, can be considered peripheral opiate receptor antagonists.

PROCEDURE

Laryngeal resistance experiments are carried out in male Sprague Dawley rats weighing 340–360 g and anesthetized with urethane (900 mg/kg, i.p.) The animals are secured in supine position and the left femoral artery and the left and right femoral veins are cannulated for recording of arterial blood pressure and administration of drugs. A right-angle polyethylene cannula (ID 1.67 mm, OD 2.42 mm) is inserted into the caudal trachea at the level of the manubrium for measuring tracheal flow via a small animal pneumotachograph. Care has to be taken to avoid damaging surrounding blood vessels or adjacent bilateral laryngeal nerves. Tracheal air flow is continually sampled (300 ml/min) for the breath to breath analysis of end tidal carbon dioxide with an infrared gas analyzer.

Laryngeal resistance in the rat is determined using a modification of the methods described by Stransky et al. (1973), Bartlett et al. (1973), and Willette et al. 1982b). The rostral portion of the trachea is cannulated with a right-angle polyethylene tube (ID 1.67 mm, OD 2.42 mm). This cannula is carefully advanced towards the larynx and secured with a suture (4-0 silk). A constant flow ($V = 30$ ml/min) of compressed air, maintained with a flow meter, is delivered through a cannula. Pre-laryngeal pressure is measured with a needle tipped pressure transducer inserted into the lumen of the flow cannula. A 1.75-cm segment of polyethylene tubing (OD 8.4 mm, ID 4.6 mm) is placed into the mouth to retract resistive components in the nasopharyngeal region.

Laryngeal resistance (*LR*) is calculated by the following equation:

$$LR = (P_L P_i - P_L P_0) / V$$

where $P_L P_i$ is the laryngeal pressure in the cannula directing a constant flow (V) through the larynx. $P_L P_0$ is the pressure in the cannula when it is removed from the trachea.

The agonist fentanyl is administered through the left femoral vein at a dose of 12 µg/kg which is equivalent to 1.5 times the ED_{99} in the conscious rat tail flick assay. The enkephaline analogue [D-Ala^2-Met^5]-enkephalinamide is injected at a dose of 250 µg/kg which acts peripherally and increases laryngeal resistance (Willette 1982a). The opiate receptor antagonists are administered similarly into the right femoral vein.

At the conclusion of the experiment, the pulmonary afferent stimulant, phenyldiguanide (25 µg/kg, i.v.) is injected into the right femoral vein to elicit laryngospasm and to determine the viability of the preparation.

EVALUATION

All summary values are expressed as the mean plus or minus the standard error of the mean (SEM) Comparisons are made with independent and paired two-tailed *t*-tests.

MODIFICATIONS OF THE METHOD

Inagi et al. (1998) assessed the effect of botulin toxin in the rat larynx be measurement of the optical density of PAS-stained laryngeal muscle after electrical stimulation, spontaneous laryngeal muscle activity, and laryngeal movement.

O'Halloran et al. (1994) studied the effects of upper airway cooling and CO_2 on breathing and on laryngeal and supraglottic resistance in anesthetized rats.

González-Barón et al. (1989) studied the modifications of larynx resistance changing bronchial tone in cats evoked by intravenous administration of 10 µg/kg carbachol as bronchoconstrictor and by fenterol (10 µg/kg) or isoproterenol (0.1 mg/kg) as bronchodilators.

Wang et al. (1999) developed an isolated, luminally perfused laryngeal preparation in anesthetized paralyzed cats in order to compare the effects of solutions with varying levels of pH and pCO_2 on pressure-sensitive laryngeal receptor sensitivity.

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D.2.2.8 Safety pharmacology of the respiratory system

PURPOSE AND RATIONALE

Regulatory agencies (EMA 1997, 2000) have pointed out the necessity to perform safety pharmacology studies before application of a new drug to human beings and to assess most carefully any potential side effects. Organs or systems acutely necessary for life, i.e., the cardiovascular, respiratory and central nervous systems, are considered to be the most important to assess in safety pharmacology studies.

Murphy (1994) described techniques and study design for safety pharmacology of the respiratory system. The potential for drugs to cause secondary or toxicologic effects that influence respiratory function were evaluated. Changes in respiratory function can result either from alterations in the pumping apparatus that controls the pattern of pulmonary ventilation or from changes in the mechanical properties of the lung that determine the transpulmonary pressures (work) required for lung inflation and deflation. Defects in the pumping apparatus are classified as hypo- or hyperventilation. The ventilatory parameters include respiratory rate, tidal volume, minute volume, peak (or mean) inspiratory flow, peak (or mean) expiratory flow, and fractional inspiratory time. Defects in mechanical properties of the lung are classified as obstructive or restrictive disorders and can be evaluated in animal models by performing flow-volume and pressure-volume maneuvers. The parameters to detect airway obstruction include peak expiratory flow, forced expira-

tory flow at 25 and 75% of forced vital capacity, and a timed forced expiratory volume, while the parameters used to detect lung restriction include total lung capacity, inspiratory capacity, functional residual capacity, and compliance. Measurement of dynamic lung resistance and compliance, obtained continuously during tidal breathing, is an alternative method for evaluating obstructive and restrictive disorders, respectively, and is used when the response to drug treatment is expected to be immediate (within minutes post-dose). The species used in studies of safety pharmacology are the same as those used in toxicology studies (mostly rats and dogs) since usually phamacokinetic data are available from these species.

Several methods useful to determine safety pharmacology on respiratory system in small animals are described in the literature.

Amdur and Mead (1958) developed a technique by which tidal volume, intrapleural pressure and rate of flow of gas in and out of the respiratory system can be measured simultaneously in unanesthetized guinea pigs for periods of several hours.

King (1966) used a nitrogen closed-circuit method for the determination of functional residual capacity in anesthetized and tracheostomized rats.

Palecek (1969) developed a method for measurement of the following ventilatory parameters in the rat: tidal volume, frequency, functional residual lung capacity, and lung resistance and compliance.

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D.2.2.8.1 Effects of respiratory depressant drugs in conscious rats

PURPOSE AND RATIONALE

Murphy et al. (1995) published a non-invasive method for distinguishing central from peripheral nervous system effects of respiratory depressant drugs in conscious rats using gas and sodium cyanide exposures and carotid body denervation.

PROCEDURE

Male Sprague Dawley rats weighing 270–490 g are used. For gas and sodium cyanide exposures the rats are exposed to gas mixtures using a two chambered plethysmograph. The body of the animal is enclosed in one chamber that is used to measure ventilatory parameters, while the head is enclosed in a cylindrical plastic headpiece attached to the body chamber. A rubber collar around the neck of the animal separated the two chambers. Specified gas mixtures are delivered from compressed gas tanks to the head chamber at a rate of 2 l/min. The type of gas mixture is controlled by a multi-port stainless steel manifold with individual valve controls.

The gas mixture contains either normal breathing air (21% O_2, 79% N_2) or elevated CO_2 (8% CO_2, 21% O_2, 71% N_2). The animals are first exposed to the normal air mixture for 5–10 min and then to the elevated CO_2 mixture for 5 min. The mean value for the 5 min CO_2 exposure is calculated for each ventilatory parameter. Following CO_2 exposure, rats are exposed to normal air mixture for a period of 5 in or until the ventilatory parameters return to normal before sodium cyanide is injected. Sodium cyanide (300 µg/kg) is administered as a bolus i.v. injection using a catheter implanted into a tail vein. The peak change in each ventilatory parameter is measured during the 1–2 min period of ventilatory stimulation following sodium cyanide injection.

For carotid body denervation, the rats are anesthetized with a cocktail containing ketamine (26.9 mg/kg), xylazine (2.9 mg/kg) and acepromazine (0.3 mg/kg). A midline incision in the neck is made and the glossopharyngeal nerve on each side of the neck is isolated. Using a dissecting microscope, the glossopharyngeal nerve is cut cranial to the carotid sinus nerve. The rats are allowed to recover 4–7 days prior to use.

Changes in ventilatory parameters are measured in conscious restrained rats using a body plethysmograph chamber with an internal volume of 2.2 l that seals around the neck of the animal and encloses the body within the chamber. Airflow rates within the chamber are measured using a pneumotach port and a differential pressure transducer attached to the plethysmograph chamber. The pressure signal is analyzed using an Upper Airway Monitor (Buxco Electronics. Inc., Sharon, CT) and values for tidal volume, respiratory rate, minute volume, peak expiratory flow, inspiratory time and expiratory time are determined for each breath. Average values of these parameters are calculated and expressed as digital output every 0.1 min using an analog-digital convertor. Mean inspiratory flow (respiratory drive) is calculated by dividing tidal volume by inspiratory time,

while fractional inspiratory time is calculated by dividing inspiratory time by the sum of inspiratory and expiratory times. Continuous tracings of flow and volume changes are also recorded to evaluate qualitative changes in ventilatory patterns.

EVALUATION

Group means and standard errors of the means (SEM) are presented for all quantitative data. Mean values of treated groups are considered to be statistically different from the control group if the calculated P value is < 0.05.

MODIFICATIONS OF THE METHOD

Drazen (1984) discussed the physiological basis and interpretation of indices of pulmonary mechanics and the rationale upon which effects of an intervention on the lung periphery can be distinguished from those of more central airways.

O'Neil and Raub (1984) described in detail pulmonary function testing in small laboratory animals.

Murphy et al. (1993) investigated the effects of intravenous injections of adenosine agonists and antagonists on pulmonary ventilation in conscious male Sprague Dawley rats.

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D.2.2.8.2 Pulmonary function in unanesthetized dogs

PURPOSE AND RATIONALE

Mauderly (1974) described analysis of pulmonary function in the unanesthetized Beagle dog.

PROCEDURE

Purebred male or female Beagle dogs at an age between 12 months and 4 years are used. The dogs are fitted with a face mask and esophageal balloon catheter (Dubin and Morrison 1969) and placed in restraining stocks (Mauderly et al. 1971) which hold them in a standing position but do not place pressure on the thorax or abdomen. This allows the inhalation of a choice of gas mixtures, collection of various fractions of the expirate, and analysis of gas samples. Airflow signals from a pneumotachograph and transpulmonary pressure signals from the esophageal catheter transducer are relayed through a preamplifier to a resistance/compliance computer containing an integrator for obtaining tidal volumes from airflow signals, an oscilloscope output for displaying breathing loops, and two subtract circuits which allow the measurement of dynamic pulmonary resistance and compliance by the hysteresis loop closing technique. The dogs are then switched at end expiration to breathing oxygen and the open circuit nitrogen washout method is used for measuring functional residual capacity. The nitrogen clearance equivalent is calculated by dividing the liters of oxygen expired during the washout by the functional residual capacity in liters. Specific compliance is calculated by dividing compliance by the functional residual capacity. The respiratory frequency, tidal volume, and minute volume during oxygen breathing are also calculated from the washout data. The dogs are then breathed 500 ppm carbon monoxide in air, and the steady state, end tidal method (Mauderly 1972) is used for measuring CO diffusing capacity and fractional uptake of CO.

For a second series of experiments, the dogs are fitted with the breathing mask, manually restrained in supine position on a contoured table, and connected to the test apparatus. Breath-by breath tensions of O_2 and CO_2 are continuously monitored at the breathing valve and recorded. When the dogs are relaxed and their breathing traces stabilized, a timed collection of the expirate is made, and a sample of arterial blood is drawn from the femoral artery. Analysis of blood samples yields arterial P_{O_2}, P_{CO_2}, pH, and hemoglobin content. The breathing traces allow calculations of the respiratory frequency and alveolar (end tidal) P_{O_2} and P_{CO_2}. Expired volume and gas tension allow calculation of the minute volume, tidal volume, O_2 uptake, CO_2 output, and respiratory exchange ratio. The percentage of alveolar, effective, and alveolar deadspace ventilation are calculated from the CO_2 output, alveolar P_{CO_2}, and arterial P_{CO_2} (Luft and Finkelstein 1968). Specific ventilation is calculated by dividing the minute volume by the O_2 uptake.

Gas samples are analyzed for N_2, O_2 and CO_2 by a mass spectrometer, and CO tensions are determined by an infrared analyzer.

EVALUATION

Student's t-test is applied to determine the statistical significance of differences between mean values of the groups.

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D.3 Antitussive activity

D.3.0.1 Antitussive activity after irritant inhalation in guinea pigs

PURPOSE AND RATIONALE

Cough is thought to be caused by a reflex. The sensitive receptors are located in the bronchial tree, particularly in the bifurcation of the trachea. These receptors can be stimulated mechanically or chemically, e.g., by inhalation of various irritants. Nerve impulses then activate the cough center in the brain. Several animals species and several irritants have been used, most frequently the citric acid induced cough in guinea pigs (Charlier et al. 1961; Karlsson et al. 1989 Braga et al. 1993).

PROCEDURE

Guinea pigs of either sex weighing 300–400 g are used. The animal is placed in a cylindrical glass vessel, with 2 tubes at either ends. One serves as the entrance of the aerosol, the other for its efflux. The latter tube has a side-arm connecting to a tambour, from which changes in pressure can be registered. A pinch-clamp with a variable screw is placed on the efflux tube beyond the side arm, permitting the regulation of the sensitivity of the system, so that the normal respiration is not registered, while the displacement of air in the enclosure caused by coughing of the animal is registered. The guinea pig is exposed to the aerosol of 7.5% citric acid in water for 10 min. Each animal is tested first to obtain the control response. The number of tussive responses is registered. One hour later, the test substance is applied either s.c. or orally, and 30 min later the guinea pig is subjected to the aerosol again. The number of coughs during 10 min is recorded.

EVALUATION

The number of coughs after treatment is expressed as percentage of the control period. Using various doses, ED_{50} values can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The citric acid induced coughing in guinea pigs has been proven to be an effective method to test antitussive agents.

MODIFICATIONS OF THE METHOD

Püschmann and Engelhorn (1978) studied the inhibition of the coughing reflex induced by inhalation of a citric acid spray in *rats*.

Other irritants have been used to induce cough, e.g. ammonia in dogs, guinea pigs, and cats (Rosiere et al. 1956; Källqvist and Melander 1957; Chen et al. 1960; Sallé and Brunaud 1960; Ellis et al. 1963), or nebulized sulfuric acid or sulfur dioxide in guinea pigs, rats, cats or dogs (Eichler and Smiatek 1940; May and Widdi-combe 1954; Winter and Flakater 1952, 1954; Friebel et al. 1955; Reichle and Friebel 1955; Wiedemeijer et al. 1960; Chermat et al. 1966; Karttunen et al. 1982). Capsaicin aerosol was used by Forsberg and Karlsson (1986), Gallico et al. (1997).

Winter and Flakater (1955) exposed sensitized guinea pigs to aerosol of a specific antigen.

Kamei et al. (1989) induced cough in rats by a nebulized solution of capsaicin. The cough reflex was measured as airflow into or out of the chamber of a body plethysmograph by a pneumotachometer head.

Carotis sinus excitation in dogs induced by injection of lobeline resulting in coughing could be suppressed by codeine (Gross 1957).

Sanzari et al. (1968) induced cough in cats anesthetized with α-chloralose by intravenous injection of 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), a ganglionic stimulant which is more potent than lobeline and possesses only marginal ganglionic blocking properties. The number of coughs was found to be a linear function of the dose of the irritant. Coughs were recorded as spikes superimposed on the respiratory pattern. The method is suitable for quantitative evaluation of antitussive activity.

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D.3.0.2
Cough induced by mechanical stimulation

PURPOSE AND RATIONALE

Cough can be induced by mechanical stimulation of the trachea in anesthetized guinea pigs (Takagi et al. 1960; Gallico et al. 1994).

PROCEDURE

Male guinea pigs weighing 350–400 g are maintained in conditioned quarters (temperature 21 ±2 °C, relative humidity 55 ±10%, 12 h on-12 h off light cycle) with food and water ad libitum for at least 1 week before use.

After overnight fasting with water ad libitum, the guinea pigs are lightly anesthetized with 25% urethane (4 ml/kg i.p.) which induces surgical levels of analgesia without depressant effects on respiratory function. Analgesia is monitored throughout the experiment as the disappearance of head shaking in response to ear pinch. The animals are maintained at a constant body temperature of 37 °C by means of a heated plate. A thin steel wire is gently inserted into the trachea through a small incision near the cricoid cartilage. Coughs are evoked by pushing the steel wire to reach the bifurcation of the trachea 35 and 5 min before oral drug administration and 30, 60 and 120 min after treatment. One violent cough occurs upon each stimulation. Only those animals that respond to both mechanical stimulations before dosing are selected and then randomly assigned to receive the test drug at various doses or the standard (codeine 15, 30 and 60 mg/kg). Ten animals per dose are used.

EVALUATION

Evaluation of the statistical significance of the results is performed with Student's *t*-test for paired data. ED_{50} values are determined by logit transformation.

MODIFICATIONS OF THE METHOD

Several other ways of mechanical stimulation have been used, e.g., by a nylon-bristled stimulator thrust into the trachea in **dogs** (Kasé 1952, 1954), or by a silver thread in **decerebrated guinea pigs** (Lemeignan et al. 1966), or by vibration of an iron slung in the trachea of a dog induced by an electromagnet (Tedeschi et al. 1959) or electrical stimulation of the trachea via a bronchoscope (Gross et al. (1958) or through implanted copper elec-

trodes (Stefko and Benson 1953; Benson et al. 1953; Granier-Doyeux et al. 1959; Stefko et al. 1961).

Hara and Yanaura (1959), Yanaura et al. (1974, 1982) induced cough in unrestrained animals after implantation of electrodes in the trachea.

Combined mechanical and chemical stimulation has been applied by Kroepfli (1950).

Kasé et al. (1976) studied the antitussive activity of d-3-methyl-N-methylmorphinan in conscious mongrel **dogs**. Coughing was induced by mechanical stimulation with a stimulator consisting of 5 hog bristles on the mucosa of tracheal bifurcation through a chronically-built tracheal fistula and in lightly anesthetized cats with a stimulator consisting of 5 whiskers of a rabbit.

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D.3.0.3 Cough induced by stimulation of the nervus laryngicus superior

PURPOSE AND RATIONALE

The probable pathways in the cough reflex arc are receptors in the area of the trachea and the large bronchi, afferent nerves mainly in the branches of the vagus nerve, a "cough center" located in the medulla oblongata, and efferent nerves closing the glottis and reinforcing the expiratory thrust. Stimulation of the Nervus laryngicus superior induces coughing. Antitussive agents with predominantly central action suppress the coughing reflex.

PROCEDURE

Cats of either sex weighing 2–3 kg are anesthetized with 40 mg/kg i.p. pentobarbital, placed on a heated operating table and their extremities secured. Since deep anesthesia suppresses coughing the dose of pentobarbital has to be adjusted. The fur is shaved ventrally at the neck. Small incisions are made at both sides of the larynx. The superior laryngeal nerves (forming a loop) are prepared carefully. After a median skin incision, the trachea is exposed and cannulated. The cannula is connected with a Fleisch-tube (size 00). One femoral artery is cannulated for registration of blood pressure via a Statham pressure transducer. One femoral vein is cannulated for intravenous application of test substances. Small hook electrodes are attached to each laryngeal nerve. At the end of an inspiration square wave impulses with a frequency of 50 Hz, an impulse width of 0.5 ms, an amplitude of 0.2–1.0 Volt, and a duration of 1–10 s are applied every 5 min. The intensity of the forced expiration is measured by the Fleisch pneumotachograph and recorded simultaneously with blood pressure on a polygraph. Prior to the intravenous application of the test compound, the response to three stimuli is recorded serving as control. After injection of the test compound or the standard the stimuli are repeated every 5 min. Suppression or diminution of the forced expiration is recorded over 1 h. Then, the next dose or the standard (codeine phosphate 1–2 mg/kg i.v.) is applied.

EVALUATION

Total or partial suppression of the forced expiration are recorded over time and expressed as percentage of control. Intensity and duration of the effect are compared with the standard.

CRITICAL ASSESSMENT OF THE METHOD

The method described by Domenjoz (1952) is very useful to detect centrally active antitussive agents like

codeine, but by definition can not determine compounds which act on cough receptors in the bronchial area. Moreover, even light anesthesia influences the cough reflex.

MODIFICATIONS OF THE METHOD

Several other assays have been described which elicit the cough reflex by central or nerve stimulation. Toner and Macko (1952) also stimulated the superior laryngeal nerve in anesthetized cats to induce a definite cough as indicated by rapid contractions of the abdominal musculature.

Mattalana and Borison (1955), Chakravarty et al. (1956) used decerebrated cats to study the central effects of antitussive drugs on cough and respiration. Cough responses were obtained by electrical stimulation of the dorsolateral region of the medulla with bipolar needle electrodes oriented by means of a stereotactic instrument.

Lindner and Stein (1959) evaluated derivatives of diphenyl-piperidono-propan, a series of antitussive drugs using a modification of the method originally described by Domenjoz (1952).

Schröder (1951) and Bobb and Ellis (1951) elicited cough in **conscious dogs** by stimulation of the vagus nerve in a surgically prepared skin loop. In anesthetized cats, coughs were elicited by electrical stimulation of the dorsolateral region in the upper medulla (Kasé et al. 1970).

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D.4
Effects on tracheal cells and bronchial mucus secretion and transport

D.4.0.1
In vitro studies of mucus secretion

PURPOSE AND RATIONALE

Mucus secretion has been studied in isolated tracheas from ferrets and dogs (Borson et al. 1980; Kyle et al. 1987).

PROCEDURE

Ferrets of either sex weighing 0.6 to 1.5 kg are anesthetized with sodium barbital intraperitoneally. The trachea is exposed and cannulated with a special Perspex cannula about 5 mm below the larynx. The animal is then sacrificed with an overdose of the anesthetic and the chest is opened along the midline. The trachea is exposed to the carina, cleared of adjacent tissue, removed from the animal and cannulated just above the carina. The trachea, with its laryngeal end down, is then mounted in a water-jacketed organ bath and bathed on its submucosal site with Krebs-Henseleit solution plus 0.1% glucose at 37 °C and bubbled with 95% O_2 and 5% CO_2. The lumen of the trachea remains air-filled. A plastic catheter is inserted into the lower cannula to form an airtight seal into which secretions can periodically be withdrawn and collected. Volumes of secretions are estimated by the weight difference of catheter lengths with and without secretions.

Simultaneous measurements of both mucus secretion and changes in tissue volume *in vitro* are achieved by mounting portions of ferret trachea cut longitudinally along the posterior wall, flattened out and pinned to a Perspex chamber. Krebs-Henseleit solution at 37 °C and gassed with 95% O_2 and 5% CO_2, is circulated on the submucosal side of the tissue, while the luminal side is exposed to the atmosphere. The surface area of the exposed tissue is about 50 mm^2. Mucus secretion is promoted by electrical field stimulation at 50–100 V, 20 Hz, 1–2 ms duration, applied through the pins holding the tissue. Before the start of each experiment, surface fluid is gently wiped off from the luminal surface with a tissue pledglet. The epithelium is coated with a layer of powdered tantalum dust; as mucus secretion from submucosal glands occurs through gland ducts, the layer of tantalum effectively traps the secreted mucus above the duct and under the tantalum layer. Nearly hemispherical hillocks are formed. The surface is photographed at intervals through a dissecting microscope and hillock diameters

are measured. Assuming the hillocks to be hemispheres, the secretion volume per unit area is calculated. Drugs are added to the submucosal bath.

EVALUATION
Secretory response after electrical stimulation in the presence or absence of drugs is recorded after 45, 90, and 135 min.

MODIFICATIONS OF THE METHOD
Quinton (1979) used isolated tracheae from cats. A segment of the trachea was mounted in a chamber such that the serosal side was constantly bathed in Ringer solution, whereas the epithelial surface was coated with water-saturated paraffin oil. Secretion was stimulated by adding appropriate drug concentrations to the bath. Under a dissecting microscope, small droplets of secretory fluid were observed to form on the tracheal epithelial surface shortly after stimulation. Timed collection of droplets secreted from three to four glands were taken up between oil blocks in constant-bore capillaries (78 µm inner diameter), and droplet volumes were measured for rate determinations usually over a period of 5 min.

CRITICAL ASSESSMENT OF THE METHODS
Both modifications of the *in vitro* methods need at least as many animals and are as time consuming as the *in vivo* methods.

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D.4.0.2 Acute studies of mucus secretion

PURPOSE AND RATIONALE
Many diseases of the respiratory tract cause both qualitative and quantitative changes in the mucus that covers and protects the airway epithelium. To study the influence of drugs, methods of collecting bronchial mucus are necessary (Braga 1988). Perry and Boyd (1941) described a method for collecting bronchial mucus from the rabbit.

PROCEDURE
Rabbits weighing 2.5 to 3.5 kg are anesthetized by intraperitoneal injection of 1.1 to 1.4 g/kg urethane. The trachea is exposed by blunt dissection and half opened, 2 cm below the cricoid cartilage. One arm of a T cannula with a large enough diameter to slightly distend the trachea is inserted into the trachea. The perpendicular arm is connected to an air outlet of a humidifier (temperature 35–38 °C, relative humidity 80%). The other arm is connected to a collection tube. The rabbit is restrained in the supine position on a 60-degree inclined board with his head downward. Respiratory tract fluids are collected in centrifuge tubes at one hour intervals. Mucus secretion can be stimulated by vagal stimulation or by ammonium chloride given by stomach tube or by pilocarpine given i.p.

EVALUATION
Time response curves after stimulants of mucus secretion are compared with data from untreated animals.

MODIFICATIONS OF THE METHOD
A method for collecting mucus from cats, using a segment of cervical trachea about 5 cm long isolated *in situ*, with nerve and blood supplies intact and a glass cannula inserted to each end, has been described by Gallagher et al. (1975).

A method to collect mucus from the upper tract trachea and the nasopharynx in dogs in acute experiments has been proposed by Proctor et al. (1973).

Engler and Szelenyi (1984) described a new method for screening mucosecretolytic compounds using tracheal phenol red secretion in **mice**. Phenol red at a dose of 500 mg/kg was injected intraperitoneally to male mice. Thirty min later, the animals were sacrificed by carbon dioxide. The whole trachea was dissected free from surrounding tissue and excised. Each trachea was washed for 30 min in 1 ml physiological saline. Afterwards, 0.1 ml 1 M NaOH was added to the washing to stabilize the pH of the lavage fluid. The concentration of phenol red was measured photometrically. Agonists were administered subcutaneously 15 min or intragastrally 30 min before phenol red was injected. Antagonists were given 5 min prior to the administration of agonists.

Other dyes, such as Evans blue or sodium fluorescein also are reported to be eliminated in the respiratory tract fluid of mice (Graziani and Cazzulani 1981).

Dye methods reported for mice can also be used for rats (Quevauviller and Vu-Ngoc-Huyen 1966). Alcian blue was used to stain the normal bronchial tree. After chronic treatment with sulfur dioxide, there were changes in bronchial coloration. Administration of drugs protected against the effects of sulfur dioxide.

Secretion from tracheal submucosal glands can be studied in **dogs** (Davis et al. 1982; Johnson and McNee 1983, 1985). In anesthetized dogs the epithelial surface of the upper trachea is exposed and coated with powdered tantalum. Secretions from the submucosal gland ducts form elevations (hillocks) in the tantalum layer. The number of hillocks that appear in a 1.2 cm^2 field is counted.

A micropipette method for obtaining secretions from single submucosal gland ducts *in vivo* in **cat** tracheas has been described (Ueki et al. 1979, 1980; Leikauf et al. 1984). In anesthetized cats an endotracheal tube was inserted into the lower trachea and connected to a constant volume respirator. The remainder of the trachea above the endotracheal tube was then dissected open by a midline incision. Paraffin oil equilibrated with HEPES buffer was then placed on the exposed mucosa to prevent drying and to aid visualization of the gland duct openings. The secretions from the gland duct openings were collected with constant-bore (99 µm ID) glass micropipettes. The volume and the viscosity of the secreted mucus were determined.

CRITICAL ASSESSMENT OF THE METHODS

For the methods of mucus collection in rabbits, cats or dogs a rather high number of animals is necessary to achieve data suitable for statistical analysis. For screening procedures the methods using dye elimination into the trachea of mice or rats seem to be preferable.

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D.4.0.3 Studies of mucus secretion with chronic cannulation

PURPOSE AND RATIONALE

Several techniques have been developed for chronic collection of mucus (Wardell et al. 1970; Yankell et al. 1970; Scuri et al. 1980).

PROCEDURE

Beagle dogs weighing 9–11 kg are anesthetized by intravenous injection of 35–40 mg/kg pentobarbital sodium. The cervical trachea is exposed by a midline skin incision and blunt dissection of the muscles. A segment approximately 10 rings in length, with an intact blood and nerve supply, is transected. The cephalic and caudal parts of the trachea are anastomosed end-to-end with interrupted gut sutures to reestablish a patent airway. The isolated segment is loosened slightly from the surrounding tissue and turned 180° to reverse cilia movement. A funnel-shaped silicone cannula is attached to the outer surface of the proximal end of the tracheal segment with surgical mesh and sutured in place. With cannulation completed, the tracheal segment is placed in a pocket below the sternohyoid muscle and the cannula brought to the surface and exteriorized through a stab wound. Alternatively, the isolated segment is closed at its caudal end with interrupted gut sutures. The mucosal surface of the cervical end of the isolated tracheal segment is sutured with interrupted silk sutures to the overlying subcutaneous tissue through a small incision in the cervical skin. Muscles and skin are sutured normally. In two or three weeks the skin heals over the small stoma resulting in

a subcutaneous pouch of functioning tracheal tissue. Mucus samples can be collected for months. In this modification, a balloon can be placed into the pouch. Pressure changes in the balloon due to contraction of the smooth tracheal muscles after physostigmine injection or vagal stimulation or relaxation after atropine injection are recorded demonstrating parasympathetic innervation.

EVALUATION

Parasympathomimetic stimulation (0.5 mg/kg pilocarpine s.c.) increases the flow rate of tracheal fluids. Pressure changes in the balloon after injection of parasympathomimetic or sympathomimetic drugs are compared with baseline values.

MODIFICATIONS OF THE METHOD

Scuri et al. (1980) inserted a T-shaped cannula into the trachea of anesthetized rabbits. The wound was sutured, the third arm was connected with a collecting tube, and after 3 days of antibiotic administration, the mucus was collected at different times to establish basal production. For the experiments, mucus was collected during a 4 h control period, then drugs were given intravenously, orally or as aerosol inhalation. Mucus was further collected during the periods of 0 to 4 and 4 to 24 h and analyzed for sialic acid, fucose, and protein content.

A tracheal pouch method in ferrets has been described by Barber and Small (1974).

Several authors published methods to determine **viscoelastic properties and rheological behavior of tracheal and bronchial mucus**: Philippoff et al. (1970), Lopez-Vidriero and Das (1977), Martin et al. (1980) Kim et al. (1982), Braga (1988), King (1988), Majima et al. (1990).

CRITICAL ASSESSMENT OF THE METHODS

The methods using pouches in dogs may be useful for physiological studies, but for pharmacological purposes the rabbit method of Scuri et al. (1980) seems preferable.

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D.4.0.4 Bronchoalveolar lavage

PURPOSE AND RATIONALE

Isolation of bronchial cells from bronchoalveolar lavage was described by Myrvik et al. (1961), Bassett et al. (1988), Fryer et al. (1994, 1997), Wang et al. (1997).

PROCEDURE

After determination of mechanical respiratory parameters in anaesthetized guinea pigs, bronchoalveolar lavage is performed via the tracheal cannula. The lungs are lavaged with 5 aliquots of 10 ml phosphate-buffered saline containing 3 mM EDTA and 100 µM isoproterenol (pH 7.2–7.4). The recovered lavage fluid (40–45 ml) is centrifuged, the cells are resuspended in 20 ml of phosphate-buffered saline, and total cells are counted using a hemacytometer. The remaining aliquot is centrifuged again and cells are stained to determine cell differentials

EVALUATION

The differences in cells recovered from bronchoalveolar lavage between treatment groups are tested by use of a one-factor analysis of variance. $P < 0.05$ is considered significant.

MODIFICATIONS OF THE METHOD

Gossart et al. (1996) determined TNF-α activity in the supernatant of bronchoalveolar lavage by the cytotoxicity against TNF-α-sensitive L929 murine fibroblasts.

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D.4.0.5 Ciliary activity

PURPOSE AND RATIONALE

Ciliary activity is a natural defense mechanism of the mucosa in the respiratory tract against harmful extraneous agents resulting in a continuous transportation of secreted mucus. Many attempts have been made to visualize and to quantify this phenomenon. Several authors used the light beam reflex method which can be used for *in vivo* as well as for *in vitro* experiments (Dalhamn 1956, 1964; Dalhamn and Rylander 1962; Hakansson and Toremalm 1963; Mercke et al. 1974; Baldetorp et al. 1976; Lopez-Vidriero et al. 1985).

PROCEDURE

Rats are anesthetized by intraperitoneal injection of tribromo-ethanol (Avertin®). The trachea is exposed and its soft parts are incised by electrocoagulation so that bleeding is avoided. The cartilaginous rings are opened sufficiently to permit microscopy. The rat is immediately placed in a moist chamber. The trachea opening is linked to a microscope (Leitz Ultropak) by means of a rubber bellows which is fitted around the lens of the microscope and is made to embrace the trachea by means of a piece of rubber tubing that is slit along its length and secured to the bellows. The beam of an illuminating lamp is concentrated to a surface of about one mm^2. By placing a heat-reflecting filter in the path of the beam the rise in temperature can be reduced. For registration of the reflected light high speed cameras with a speed of 220 exposures are used. Alternatively, the reflected light from the microscope is directed to a TV camera and amplified to be displayed on a TV screen. The frequency of ciliar beats is recorded over one hour.

EVALUATION

The beat frequency of treated animals is compared with that of controls.

MODIFICATIONS OF THE METHOD

Mercke et al. (1974) described a stroboscopic method for standardized studies of mucociliary activity in rabbit tracheal mucosa.

Lierle and Moore (1935) inserted windows into anesthetized rabbits for observation of the ciliary activity in the maxillary sinus of living animals.

With modern equipment, a similar technique has been used by Hybbinette and Mercke (1982a–c), Lindberg and Mercke (1986), Lindberg et al. (1986), Mercke et al. (1987) to study the role of several pharmacologic agents in the mucociliary defense of the rabbit maxillary sinus.

Corssen and Allen (1958) compared the toxic effects of various local anesthetic drugs on human ciliated epithelium *in vitro* by observation of rotating globes of human tracheal epithelium in tissue culture.

Cheung (1976) performed high speed cinemicrographic studies on rabbit tracheal (ciliated) epithelia.

Iravani (1967, 1971, 1975) studied the ciliary activity in the intrapulmonary airways of rats by incident light microscopy.

Lee and Verdugo (1976), Verdugo et al. (1980) recommended laser light-scattering spectroscopy for the study of ciliary activity.

Rutland and Cole (1980) and Hesse et al. (1981) used a non-invasive method for obtaining nasal ciliated epithelium which is suitable for measurement of ciliary beat frequency.

Van de Donk et al. (1980) used isolated chicken embryo tracheas to measure the effects of preservatives on ciliary beat frequency. Maurer et al. (1982) studied the role of ciliary motility in acute allergic mucociliary dysfunction in cultivated ciliated cells from sheep.

Lopez-Vidriero et al. (1985) studied the effect of isoprenaline on the ciliary activity of an *in vitro* preparation of rat trachea.

Braga et al. (1986) described a simple and precise method for counting ciliary beats directly from the TV monitor screen using specimens of human ciliated epithelium obtained by brushing the nasal mucosa.

Curtis and Carson (1992) used pieces of human nasal epithelium for computer-assisted video measurement of ciliary beat frequency *in vitro*. Ciliary beat frequency was viewed with a microscope equipped with a phase contrast objective. The microscopic image was recorded by a camera and data stored by a videorecorder. For measuring ciliary beating, tapes were displayed with amplification on a monitor. A photoelectric transducer was positioned over the video image of the cilia. Movement of the cilia interrupting the light path caused changes in light intensity recorded by the photocell transducer.

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D.4.0.6 Studies of mucociliary transport

PURPOSE AND RATIONALE

Mucus flow has been studied in *in vitro* and *in vivo* experiments (Iravani 1971; Ahmed et al. 1979). The rate of mucus flow can be estimated by measuring the time needed for certain particles to travel a known distance in the trachea. Numerous substances have been used such as charcoal particles (Dalhamn 1956), Teflon discs (Ahmed et al. 1979), and other pulverized materials (Deitmer 1989). The effect of local radioactivity on tracheal mucous velocity of sheep has been studied *in vitro* and *in vivo* by Ahmed et al. (1979).

PROCEDURE

For *in vitro* experiments, sheep are sacrificed during anesthesia, the trachea is exposed, clamped, and resected just below the cricoid cartilage. The chest is opened and the trachea is resected at the level of the carina. Then the trachea is slit open along the posterior membranous wall, pinned with gentle stretching on a board, and slanted upward at an angle of 25°. A metric ruler is placed along the board as a measuring reference. The board is then placed in a Plexiglas® chamber with a constant temperature of 37 °C and 100% humidity. Teflon discs are spread on the mucous layer. Tracheal mucus velocity is estimated by filming the movements of the radioopaque Teflon discs visualized on a television monitor connected to a camera. Disc motion is recorded for 1–2 min on a videotape, and the distance measured during the elapsed time is obtained from the videomonitor.

For *in vivo* measurements of tracheal mucus velocity, the roentgenographic method of Friedman et al. (1977), Sackner et al. (1977) is used. The sheep are restrained, and their heads are immobilized with a sling. The nasal mucosa is sprayed with a 2% lidocaine solution for topical anesthesia. A bronchofiberscope is inserted transnasally, and its tip is placed just below the vocal cords. Radioopaque Teflon discs 1.0 mm in diameter, 0.8 mm thick, and weighing 1,76 mg are blown through the inner channel of the bronchofiberscope onto the tracheal mucosa in a circumferential distribution. The cervical trachea containing the discs is visualized in the lateral projection with a television monitor. Disc motion is recorded on a videotape while the time is displayed on a digital clock. The disc image is marked to obtain the distance traveled. This distance is measured with a ruler, and the linear velocity of the disc is computed by dividing the distance by the elapsed time. This procedure is repeated for all discs in the field of view. To compute a mean tracheal mucus velocity, data from 10–15 discs are obtained in each filmed run.

EVALUATION

Disc velocities measured *in vitro* or *in vitro* are compared before and after treatment.

MODIFICATIONS OF THE METHOD

Mucociliary transport has been studied on the hard palate of decapitated frogs measuring the transport velocity of small particles, e.g., pieces of cork or charcoal (Kochmann 1930; Sadé et al. 1970).

Suzuki (1966) measured the movement of a standard object (1 mm^2 aluminum foil) on the ciliated surface of the palate of frogs.

Mucociliary clearance by the *in vitro* frog method was used by Leitch et al. (1985) to study the effects of ethanol.

Movement of poppy seeds in rabbit tracheal preparations was studied by Kensler and Battista (1966).

In chicken nasal mucosa the interaction between mucociliary transport and the ciliary beat was studied by Ukai et al. (1985).

Mucus transport in the respiratory tract of anesthetized cats was measured with uniform particles of lycopodium spores triturated with lamp black (Carson et al. 1966).

Mucociliary clearance velocities were determined by a radioisotopic method in dogs (Giordano et al. 1977, 1978).

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D.4.0.7 Culture of tracheal epithelial cells

PURPOSE AND RATIONALE

Marked morphological changes of the airway epithelium, up to severe damage, are frequently observed in inflammatory airway diseases and appear to play an important role in the pathogenesis of the broncho-obstructive symptoms (Webber and Corfield 1993; Hay et al. 1994). Freitag et al. (1996) studied the effects of lipopolysaccharides (LPS) and TNF-α on cultured rat tracheal epithelial cells and determined NO synthase activity.

PROCEDURE

Trachea of newborn rats, cut into small pieces, are explanted with the epithelial surface downwards onto 60 mm culture dishes (Lechner et al. 1985) and cultured in low-calcium (60–80 µmol/l) RPMI-1640 medium at 37 °C and 5% CO_2. The medium containing 16% fetal calf serum is supplemented with epidermal growth factor and other growth promoting factors, such as 80 ng/ml cholera toxin, 2.6 ng/ml estradiol, 180 ng/ml hydrocortisone, 2.5 µg/ml insulin, 12.5 µg/ml transferrin, and 100 U/ml penicillin, 100 µg/ml streptomycin and 3.5 µg/ml amphotericin B to allow a selective outgrow of epithelial cells (Emura et al. 1990). Confluent epithelial layers are obtained after about 6–10 weeks at which time all cells show a positive staining with a pan-cytokeratin-antibody. Confluent cells of fol-lowing passages are treated for four subsequent days with LPS (10 µg/ml) or TNF-α (500 U/ml).

The **morphology** of the cells is evaluated is evaluated by daily inspection using a phase contrast microscope with photographical documentation. Cell density in the culture is determined in each culture dish by counting daily the number of cells in four marked areas (each 2 000 μm^2). For immunocytochemical staining, the cells are fixed by incubation in 100% methanol at –20 °C for 20 min. The cells are washed with Ca/Mg-free phosphate buffered saline and several areas are marked of by nail-varnish. A solution of the anti-cytokeratin(pan)-antibody (monoclonal mouse IgG, Boehringer Mannheim) is added, incubated for 2 h at room temperature and after washing incubated for 30 min with a secondary antibody (FITC coupled, polyclonal rabbit anti-mouse IgG, Sigma). After washing, fluorescence microscopy is performed using a microscope with a FITC-specific filter combination. Unspecific fluorescence is excluded by performing the staining procedure in a different area of the same culture, but without the addition of the primary antibody.

For **determination of NO synthase activity**, confluent cultures are washed with oxygenated and prewarmed (37 °C) Krebs-HEPES medium. Then the cells are incubated for 1 h in the same medium containing 37 kBq ^{3}H-L-arginine (100 nmol/l). After collection of the supernatants the cells are extracted in 1 ml of 0.4 mol/l $HClO_4$ for 2 h at 0–4 °C.

By **HPLC analysis** ^{3}H-compounds (^{3}H-L-citrulline, ^{3}H-L-ornithine and ^{3}H-L-arginine) in incubation media and cell extracts are separated on a reverse phase column (length 250 mm, inner diameter 4.6 mm, prepacked with Shadon ODS-Hypersil, 5 mm) using as mobile phase 0.1 mmol/l sodium phosphate buffer (adjusted to pH 1.8) which contains octane sulphonic acid sodium salt (400 mg/l), Na_2EDTA (0.3 mmol/l) and methanol (6.25% v/v) with a flow rate of 1 ml/min (Hey et al. 1995). The eluate is collected in 1 min fractions into counting vials. After addition of a commercial scintillation cocktail the radioactivity is determined by liquid scintillation spectrophotometry. External standardization is used to correct for counting efficiency. The retention time is determined by the use of ^{14}C-labelled (L-citrulline or L-ornithine) or ^{3}H-labelled (L-arginine) standards. Protein content in cells is determined by a commercially available assay.

EVALUATION

The amounts of ^{3}H-L-citrulline in supernatants or cell extracts are expressed as DPM/µg protein. Changes in cell density are expressed as % of the density observed in each culture dish at the start of the experiment. Mean values are given ±SEM. The significance of difference is evaluated by Student's *t*-test.

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Chapter E
Psychotropic and neurotropic activity[1]

[1] Reviewed by W. H. Vogel, contribution of biochemical methods in the first edition by F. P. Huger.

E.1 Effects on behavior and muscle coordination

E.1.1 Spontaneous behavior

E.1.1.1 General considerations

The effects of drugs on the central and peripheral nervous systems can be easily recognized in normal animals. This does not necessarily mean that these effects can be used in therapy. Observing the global effects of drugs during LD_{50}-determinations, pharmacologists can detect psychotropic activity. Only, if these effects occur also in doses considerably below the LD_{50}, are further evaluations justified. This basic experience resulted in the development of a variety of observational tests and activity measurements.

E.1.1.2 Observational assessment

PURPOSE AND RATIONALE

A systematic, quantitative procedure assessing the behavioral state of mice for the evaluation of drugs has been described by Irwin (1964, 1968). The method is applied in the beginning of pharmacological screening to detect psychotropic activities. It allows to identify and differentiate the profile pattern of various classes of pharmacological agents. Furthermore, observational assessment allows into the safety and potential toxicity profile of a new drug.

PROCEDURE

Mice of either sex (NMRI-strain) with a weight between 18 and 22 g are kept under standard laboratory conditions 5 days before the experiment. Animals tested for oral administration have water ad libitum but are deprived of food 16 h before the test. Animals for intravenous or intraperitoneal administration have access to food and water ad libitum until the test. For unknown substances the following doses are given:

- i.v. administration: 100, 200, 400 mg/kg
- i.p. administration: 300, 600, 1 200 mg/kg
- oral administration: 500, 1 000, 2 000 mg/kg

Three animals are used for each dose. One group of 3 animals receiving the vehicle only, serves as control group.

Immediately after drug administration, the animals are closely observed for 2 h (following i.v. or i.p. administration) and for 5 h (following oral administration) by a "blind" observer. The following parameters are checked and compared to the vehicle control group:

Effects on CNS	Effects after manipulations
spontaneous motor activity	auditory stimulus response
restlessness	escape after touch
grooming behavior	righting reflex
squatting	paresis of hind limbs
staggering	paresis of forepaws
ataxic gait	catalepsy in induced positions
lying flat on the belly	
lying flat on the side	**Effects on reflexes**
lying flat on the back	
sleeping	pinna reflex
narcosis	corneal reflex
bizarre behavior	pain following stimulation
timidity	
Straub´s phenomenon	**Effects on autonomic nervous system**
writhing	
tremors	pupil diameter (constriction or dilatation)
twitches	eyelids (closure or exophthalmus)
opisthotonus	secretion of sweat
clonic convulsions	salivation
tonic convulsions	lacrimation
rolling and jumping convulsions	cyanosis
	piloerection
	defecation
	urination

Depending on the route of administration, the observations are performed at different time intervals: after intravenous and after intraperitoneal administration during the first 30 min, and after 1 and 2 h; after oral administration during the first 60 min, and after 2 and 6 h. The number of deaths is counted during the first 24 h and after 7 days in order to evaluate acute and late toxicity. Arbitrary scores are chosen for each symptom. If positive effects are seen with the lowest dose, the experiment is repeated with lower doses being decreased by a factor of 3.

CRITICAL ASSESSMENT OF THE TEST

The test has been used by almost every laboratory in the world involved in screening of potential new drugs. Almost every laboratory has introduced its own modifications. In particular, the scores and the calculations differ from one laboratory to the other. Additional tests, such as grip strength or rotarod, described below, have been included into the primary screen. Graph-bar profiles have been established for known drugs in order to rate new substances accordingly. The test is definitively an useful tool for primary screening resulting in

hints on psychotropic activity but also for actions on other systems. Nevertheless, this test can not substitute for more sophisticated tests for the evaluation of psychotropic activity.

MODIFICATIONS OF THE METHOD

Based on the guidelines of the United States Environmental Protection Agency (USEPA) (1991) Mattsson et al. (1996) described a performance standard for clinical and functional observational battery (FOB) for examinations of rats. The performance standard was an idealized composite of FOB data from experienced laboratory personnel, each person tested on a separate set of four groups of rats. The rats were examined in random order and treatments were either (a) saline, (b) chlorpromazine, (c) atropine, followed by physostigmine or (d) amphetamine.

Testing of neurotoxicity with new pharmaceuticals was reported by Haggerty (1991). He proposed a primary screen for rodents, consisting of a functional observation battery and an automated test for motor activity. In addition, a functional observation battery for dogs was developed. The rat tier I screen assesses such functions as home cage and open field activity, stimulus reactivity, and neuromuscular function. The dog tier I screen emphasizes evaluation of gait, postural reactions, and reflex function.

Rambert (2000) underlined the importance of some general conditions for general pharmacology of the CNS, such as choice of the animal species, administration route and controls of the experimental context (surroundings, temperature, schedules of the trials, interference with food-intake episodes, influence of noises and smells, expertise of the experimental staff).

Crawley and Paylor (1997), Crawley (2000) proposed a test battery and behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. Several neurological and neuropsychological tests are described which can be used as first screen for behavioral abnormalities in mutant mice. Multiple behavioral paradigms are included for several categories, such as neurological reflexes, sensory abilities, motor functions, learning and memory, feeding, sexual behavior, analgesia, aggression, anxiety, depression, schizophrenia, and drug abuse.

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E.1.1.3 Safety pharmacology core battery

PURPOSE AND RATIONALE

Regulatory agencies (EMA 1997, 2000) have pointed out the necessity to perform safety pharmacology studies before application of a new drug to human beings and to assess most carefully any potential side effects. Organs or systems acutely necessary for life, i.e., the cardiovascular, respiratory and central nervous systems, are considered to be the most important to assess in safety pharmacology studies. The following parameters of CNS activity should be assessed appropriately: motor activity, behavioral changes, coordination, sensory/motor reflexes and body temperature.

Detailed descriptions of tests are found for

Observation tests:

E.1.1.2 Observational assessment

Motor activity and behavior:

E.1.2.2 Method of intermittent observations
E.1.2.3 Open field test
E.1.2.4 Hole-board test
E.1.2.5 Combined open field test

Coordination:

E.1.3.1 Inclined plane
E.1.3.2 Chimney test
E.1.3.3 Grip strength
E.1.3.4 Rotarod method

Sensory/motor reflexes:

E.1.3.5 Influence on polysynaptic reflexes

E.1.3.6 Masticatory muscle reflexes

Follow-up studies for CNS safety pharmacology are recommended, such as behavioral pharmacology, learning and memory, specific ligand binding, neurochemistry, visual, auditory and/or electrophysiology examinations. Detailed descriptions of these tests are found in the respective chapters, e.g.,

E.1.2.6 EEG analysis from rat brain by telemetry.

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E.1.2 Effects on motility (sedative or stimulatory activity)

E.1.2.1 General considerations

A survey on methods to evaluate depressants of the central nervous system has been published by Turner (1965). Many of these tests are still valid in spite of the fact that new classes of drugs have been introduced since that time which have not only augmented the therapeutic armamentarium but also changed the battery of pharmacological tests.

Sedative properties of drugs are tested mostly in mice or rats. Their spontaneous motor activity depends on various factors, such as the social situation (one or more animals), familiarity with the test environment, light and temperature. The term spontaneous motor activity includes different types of movements, such as locomotion, rearing, sniffing, grooming, eating and drinking. These phenomena can be well recognized by a skilled observer, but are difficult to record over long periods of time and to quantitate. Therefore, besides procedures based on observation many methods for automatic registration have been developed. Almost every pharmacologist working in this field has designed his/her own apparatus. Several attempts have been made to measure not only simple locomotion, but also rearing and other types of movement. The conditions to characterize drug effects by measuring locomotor activity have been surveyed by Kinnard and Watzman (1966), Geyer (1990).

A special phenomenon, called "thigmotaxis" which means that rats have the tendency to remain close to the walls of the cage, has been described by Barnett (1963). Moreover, methods to measure curiosity have been recommended. Only a few examples of prototypic methods and equipment can be given.

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E.1.2.2 Method of intermittent observations

PURPOSE AND RATIONALE

The method described by Ther (1953) was designed to study stimulant and sedative drugs. The use of 3 mice per group implied a special social situation of the animals. For testing sedative activity, the mice are additionally treated with a stimulant, and for testing stimulant activity with a sedative drug. Each group of mice is observed repeatedly only for a short period of time during one hour and compared to a control group by a "blind" observer.

PROCEDURE

Mice of either sex with an average weight of 25 g are deprived of food and water for 24 h before the test. To avoid any influence of the circadian rhythm the experiment is performed only between 8:00 and 12:00 A.M. Twelve animals are divided into groups of 3 mice. One group of 3 mice serves as control group, the other groups receive different doses of the test drug intraperitoneally. For testing sedative activity, the mice are injected after 10 min subcutaneously with 0.5 mg/kg methamphetamine. For testing stimulant activity, the mice are treated with 800 mg/kg paraldehyde. Ten minutes afterwards, each group is placed into a glass jar of 12 cm diameter and 20 cm height which in turn are

placed in a wooden box of 130 × 50 × 30 cm. The glass jars are illuminated from above. Ten minutes after administration of methamphetamine or paraldehyde the observation is started.

EVALUATION

During 1 h the observer looks every minute for 1 s to each jar and registers if none, one, two or all three mice show any characteristic change in locomotion, rearing, grooming or sniffing. The maximum count in 1 h would be 180. Generally, activity decreases within 1 h. Nevertheless, methamphetamine treated animals have a total count between 120 and 150. Groups treated with an effective sedative drug show a dose dependent decrease of total counts. The number of counts in the treated groups is calculated as percentage of controls. From dose-response curves ED_{50}-values can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method gives reliable results for sedative drugs and for compounds with central depressant activity, such as antihistaminics, neuroleptics and hypnotics. The disadvantage of the method lies in the fact that a skilled and trained observer is needed in order to get reproducible results. Therefore, several attempts have been made to automatize the method of intermittent observation.

MODIFICATIONS OF THE METHOD

Schaumann and Stoepel (1961) followed the principle of intermittent observation using a camera mounted above small wire cages (10 × 10 cm). The mice in the cages were photographed with an exposure time of 3 s every 7,5 min over a period of 2,5 h. Mice without movements give clear pictures, calculated as zero. Slight movements induce blurred contours, calculated as 1 point, and major movements give completely blurred pictures, calculated as 2 points. Normal mice show 20 or less points during 20 observations. Mice with more than 20 points are considered to be stimulated, mice with less than 5 points to be sedated. In this way ED_{50}-values can be calculated.

Vogel and Ther (1963) published an apparatus with automatic registration of intermittent observations. 18 cages are used which have a freely movable bottom with minimal weight which is supported by springs. A small permanent magnet attached below the floor induces an electrical current in a coil if the bottom is moved. Two mice are brought into each cage. The control device registers during a variable period between 0.5 and 3 s if the bottom of one cage is moved or not. Within one minute, all 18 cages are registered successively. The number of movements within one hour is recorded for each cage. The sensitivity of the electric induction system is variable. A calibration is possible which picks up every movement of the animal, however, does not register the movements due to breathing. Dose-response curves can be established for stimulant and sedative drugs.

Meyer (1962) measured the time until the exploration motility decreased to one third of the initial value.

Koek et al. (1987) used the principle of intermittent observations for various activities of rats such as locomotion, rearing, sniffing, licking, gnawing, grooming, loss of righting, Straub tail, ect. in order to compare the behavioral effects of drugs.

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E.1.2.3 Open field test

PURPOSE AND RATIONALE

Interruption of light beams as a measure of movements of rats or mice in a cage ("open field") has been used by many authors such as Dews (1953), Saelens et al. (1968) Nakatsu and Owen, (1980). Recently developed devices allow to register not only general motor activity but also locomotion, rearing and the speed of locomotion (Barros et al. 1991; Ericson et al. 1991).

PROCEDURE

The rats are observed in a square open field arena (68 × 68 × 45 cm) equipped with 2 rows of 8 photocells, sensitive to infrared light, placed 40 and 125 mm above the floor, respectively. The photocells are spaced 90 mm apart and the last photocell in a row is spaced 25 mm from the wall. Measurements are made in the dark in a ventilated, sound-attenuating box. Interruptions of photocell beams can be collected by a microcomputer and the following variables can be evaluated:

- Motor activity: All interruptions of photo beams in the lower rows.
- Peripheral motor activity: Activation of photo beams in the lower rows, provided that the photo-beams spaced 25 mm from the wall were also activated.
- Rearing: All interruption of the photo beams in the upper rows.
- Peripheral rearing: Interruption of photo beams in the upper rows, provided that the photo beams spaced 25 mm from the wall were also activated.
- Locomotion: Successive interruptions of photocells in the lower rows when the animal is moving in the same direction.
- Speed: The time between successive photo beam interruptions during locomotion collected in 0.1 s categories.

Adult male Sprague-Dawley rats with a weight between 280 and 320 g are used. Drugs are injected subcutaneously 10 to 40 min. before test. The rats are observed for 15 min whereby counts per min. are averaged for 3 min intervals.

EVALUATION

Dose-response curves can be obtained for sedative and stimulant drugs, whereby the various parameters show different results. The effects of various doses are compared statistically with the values of controls and among themselves.

CRITICAL ASSESSMENT OF THE METHOD

Measurement of several parameters in an open field device allows to differentiate between various types of sedative or stimulant drugs, but these differences can only be detected if dose-response curves are obtained for each parameter.

MODIFICATIONS OF THE METHOD

Besides interruption of light beams, devices based on capacitance systems such as Animex® (Columbus Instruments, Ohio, USA) and Varimex®, have been developed and are widely used (Crunelli and Bernasconi 1979; Liu et al. 1985; Laviola G and Alleva E 1990; Honma et al. 1990; Magnus-Ellenbroek et al. 1993; Dauge et al. 1995; Petkov et al. 1995; Surmann et al. 1995; Gillies et al. 1996; Irifune et al. 1997; Ghelardini et al. 1998).

Nikodijevic et al. (1991) studied the behavioral effects of A_1- and A_2-selective adenosine agonists and antagonists in mice using a Digiscan activity monitor (Omnitech Electronics Inc., Columbus, OH) equipped with an IBM-compatible computer. The monitor included multiple activity monitor cages ($40 \times 40 \times 30.5$ cm), each of which was surrounded by horizontal and vertical sensors not detectable by the rodent.

Steiner et al. (1997) found that D_3 receptor deficient mice enter the center of an open field significantly more than their littermates suggesting an anxiolytic-like effect of the D_3 receptor mutation.

Vorhees et al. (1992) described a locomotor activity system for rodents. The system consists of a black, ventilated test chamber, internally lighted with a ceiling mounted video camera. The camera's image is transmitted to a contrast-sensitive tracker which maps the point of highest contrast and relays the digitalized coordinates to a PC. Dedicated software stores the information and simultaneously displays a map of the tracked subject.

Rex et al. (1996, 1998) described a modified open field test sensitive to anxiolytic drugs. Food-deprived rats were placed in one corner of the open field containing food in the center. The number of rats beginning to eat within the first 5 min was increased by known anxiolytic drugs.

Several computerized systems, based on interruptions of infrared light beams, on magnetic field or on video-analysis have been developed by Technical and Scientific Equipment GmbH, D-61348 Bad Homburg, Germany, or by Bilaney Consultants Ltd., St Julians, Sevenoaks, Kent, UK.

A novel method for counting spontaneous motor activity in rats was proposed by Masuo et al. (1997). In the "Supermex" system, a sensor detects the radiand body heat of an animal.

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E.1.2.4 Hole-board test

PURPOSE AND RATIONALE

The evaluation of certain components of behavior of mice such as curiosity or exploration has been attempted by Boissier et al. (1964) and Boissier and Simon (1964). They used an open field with holes on the bottom into which the animals could poke their noses. The "planche à trous" or "hole-board" test has become very well recognized and has been modified and automatized by many authors.

PROCEDURE
Mice of either sex (NMRI strain) with a weight between 18 and 22 g are used. The hole-board has a size of 40 × 40 cm. Sixteen holes with a diameter of 3 cm each are distributed evenly on the floor. The board is elevated so that the mouse poking its nose into the hole does not see the bottom. Nose-poking is thought to indicate curiosity and is measured by visual observation in the earliest description and counted by electronic devices in more recent modifications. Moreover, in the newer modifications motility is measured in addition by counting interruption of light beams. Usually, 6 animals are used for each dose and for controls. Thirty minutes after administration of the test compound the first animal is placed on the hole-board and tested for 5 min.

CALCULATION
The number of counts for nose-poking of treated animals is calculated as percentage of control animals.

CRITICAL ASSESSMENT OF THE METHOD
Poking the nose into a hole is a typical behavior of mice indicating a certain degree of curiosity. Evaluation of this component of behavior has been proven to be quite useful. Benzodiazepines tend to suppress nose-pocking at relatively low doses.

MODIFICATIONS OF THE METHOD
A hole-poke measuring system is commercially available from Technical and Scientific Equipment GmbH, D-61348 Bad Homburg, Germany.

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E.1.2.5 Combined open field test

PURPOSE AND RATIONALE
The simultaneous determination of locomotion and curiosity by using a modification of the hole-board test and a photo-beam system has been proposed as a relatively simple test (Weischer et al. 1976). Several types of such equipment are commercially available.

PROCEDURE
Male mice (NMRI-strain) with an average weight of 30 g are used. Each animal is tested individually in an automated open-field box which consists of a black Plexiglas cage (35 × 35 × 20 cm) with a post (8 × 8 × 20 cm) in the center of the cage. Two evenly spaced photo cell beams perpendicular to the wall and 2 cm above the floor divide the box into 4 compartments. Every photo cell beam interruption is registered automatically as an activity count. Each wall of the cage contains 4 evenly spaced 2 cm diameter holes in a horizontal array 7 cm above the floor. A row of 4 photocell beams is mounted 1 cm outside of the holes and automatically records every exploratory nose-poke. Thirty min. after intraperitoneal and 60 min. after oral administration of the test compound the animal is placed into the cage and the behavior recorded for a period of 5 min. Ten mice are used for each dose as well as for controls.

EVALUATION
Counts for motility (interruption of photo cell beams inside the cage) and for curiosity (interruption of photo cell beams outside the cage due to nose-poking) are recorded individually. The mean values of the treated groups are expressed as percentage of the control group. Using different doses, dose-response curves can be obtained.

CRITICAL ASSESSMENT OF THE METHOD
A dissociation between exploratory behavior and locomotion has been found with several drugs. Even well known stimulants can reduce exploratory behavior with a concomitant increase in locomotion. Depending on the dose, tranquilizers can reduce exploration without affecting locomotion. Due to the modifications of the equipment the results of different authors are often difficult to compare.

The limitations of photocell activity cages for assessing effects of drugs were discussed by Krsiak et al. (1970).

MODIFICATIONS OF THE TEST
Geyer (1982) described a similar device and reported different effects of amphetamine, caffeine, apomorphine and scopolamine. Adams and Geyer (1982) used this device to study the LSD-induced alterations of locomotor patterns and exploration in rats.

Geyer et al. (1986) described a behavioral monitor which was designed to assess the spatial and temporal sequences of locomotor movements in rats.

Other authors, e.g. Wolffgramm et al. (1988) used video recording of the movements of mice in the evening hours at low illumination and counted the locomotor activity as the number of field crossings and the exploratory activity as the number of rearings.

Ljungberg and Ungerstedt (1977) designed a testbox for the automatic recording of eight components of behavior in rats including compulsive gnawing induced by apomorphine.

Matsumoto et al. (1990) described a system to detect and analyze motor activity in mice consisting of a doughnut-shaped cage with 36 units of detectors radially arranged from the center of the cage. Each detector unit consisted of 4 pairs of photosensors (higher-, lower-, inner-, and outer-position sensors).

Schwarting et al. (1993) described a video image analyzing system for open-field behavior which measures turning behavior, thigmotactic scanning and locomotion in rats.

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E.1.2.6 EEG analysis from rat brain by telemetry

PURPOSE AND RATIONALE

Field potential analysis of freely moving rats by radioelectroencephalography from different brain areas has been developed as a sensitive method in pharmacology (Dimpfel et al. 1986, 1989, 1990, 1992; Kropf et al. 1991).

PROCEDURE

Male adult rats are implanted with four stainless steel electrodes, mounted on a base plate, into the frontal cortex, striatum, thalamus, and reticular formation. The plate carries a microplug for a four-channel radiotransmitter. During the experimental sessions, which start 2 weeks after surgery, the transmitted field potentials are analyzed in real time using Fast Fourier Transformations. The resulting power density spectra are segmented into six frequency bands, each representing the integrated power over a certain frequency band.

EVALUATION

The data from three pre-drug periods of 15 min each are compared with those from continuously monitored 15-min postdrug periods.

MODIFICATIONS OF THE METHOD

De Simoni et al. (1990) developed a miniaturized optoelectronic system of telemetry for data obtained in freely moving animals by *in vivo* voltammetry, an electrochemical technique that uses carbon fiber microelectrodes to monitor monoamine metabolism and release continuously (Justice 1987).

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E.1.3 Tests for muscle coordination

E.1.3.1 Inclined plane

PURPOSE AND RATIONALE

The method of Allmark and Bachinski (1949) using an inclined screen was originally developed for testing curare-like agents. Later on, it has been used by many authors (e.g. Randall et al. 1961) for testing compounds for muscle relaxant activity. The principle of an inclined plane has been used by Ther, Vogel and Werner (1959) for differentiating neuroleptics from other centrally active drugs. Rivlin and Tator (1977) also used an inclined plane to assess skeletal muscle relaxation.

PROCEDURE

The plane consists of two rectangular plywood boards connected at one end by a hinge. One board is the base, the other is the movable inclined plane. Two plywood side panels with degrees marked on their surface are fixed on the base. A rubber mat with ridges 0.2 cm in height is fixed to the inclined plane which is set at 65 degrees. Male mice (Charles River strain) with a body weight between 20 and 30 g are used. The test compound or the standard are administered to groups of 10 mice either i.p. or s.c. or orally. Thirty, 60 and 90 min thereafter, the mice are placed at the upper part of the inclined plane and are given 30 s to hang on or to fall off.

EVALUATION

The peak time is determined as the time at which a compound produces the maximum performance deficit. At this time interval, a range of doses is tested using 10 animals per group. ED_{50} values are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method has been proven to be a simple assay for muscle relaxant activity. Although the muscle relaxant tests satisfy the criteria of sensitivity and relative potency compared with clinically effective doses, the effects of anxiolytics are not clearly differentiated from neuroleptics and even from neurotoxic compounds.

MODIFICATIONS OF THE METHOD

Instead of an inclined wooden board, an inclined screen has been used by Randall et al. (1961) and Simiand et al. (1989).

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E.1.3.2 Chimney test

PURPOSE AND RATIONALE

The "test de la cheminée" has been introduced by Boissier et al. (1960) as a simple test for tranquilizing and muscle relaxant activity.

PROCEDURE

Male mice (CD1, Charles River) weighing between 16 and 22 g are used in groups of 10 animals per dose. Pyrex-glass cylinders 30 cm long are required. The internal diameter varies with the animal's weight: for mice weighing 16 to 18 g, the diameter is 22 mm, for mice weighing 18 to 20 g, 25 mm; for mice weighing 20 to 22 g, 28 mm. Each tube has a mark 20 cm from its base. Initially, the tube is held in a horizontal position. At the end of the tube, near the mark, a mouse is introduced with the head forward. When the mouse reaches the other end of the tube, toward which it is pushed if necessary with a rod, the tube is moved to a vertical position. Immediately, the mouse tries to climb backwards and performs coordinated movements similar to an alpinist to pass a chimney in the mountains. This gave the name for the test. The time required by the mouse to climb backwards out at the top of the cylinder is noted.

EVALUATION

The ED_{50} (with 95% confidence limits), the dose for which 50% of the animals fail to climb backwards out of the tube within 30 s, is calculated by log-probit analysis.

CRITICAL ASSESSMENT OF THE METHOD

The chimney test can be used as an additional test with other tests determining muscle relaxant activity.

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E.1.3.3 Grip strength

PURPOSE AND RATIONALE

The test is being used to assess muscular strength or neuromuscular function in rodents which can be influenced not only by sedative drugs and muscle relaxant compounds but also by toxic agents.

PROCEDURE

The method was described as 'test de l'agrippement' by Boissier and Simon (1960). Male or female mice with an average weight of 22 g are used. In a preliminary experiment the animals are tested for their normal reactivity. The animals are exposed to a horizontal thin threat or metallic wire suspended about 30 cm into the air which they immediately grasp with the forepaws. The mouse is released to hang on with its forelimbs. Normal animals are able to catch the threat with the hind limbs and to climb up within 5 s. Only animals who fulfill this criterion are included into the experiment. Ten mice are used in the control group and in the experimental groups. After oral or subcutaneous administration the animals are tested every 15 min. Animals which are not able to touch the threat with the hind limbs within 5 s or fall off from the threat are considered to be impaired. The test is continued for 2 h. The animals are observed for their behavior in the cages. Only if their behavior and their motility in the cage seem to be normal the disturbance of the grasping reflex can be considered as caused by central relaxation.

EVALUATION

The percentage of animals loosing the catching reflex is calculated. By use of different doses, ED_{50}-values are calculated. Likewise, time-response curves can be established.

CRITICAL ASSESSMENT OF THE METHOD

Only simultaneous observation of the animals under normal conditions gives the possibility to distinguish between central relaxation and toxic effects on neuromuscular function.

MODIFICATIONS OF THE METHOD

Kondziella (1964) described a method for the measurement of muscular relaxation in mice based on the capacity of hanging from a horizontal griddle.

Meyer et al. (1979) described a technique to measure the fore- and hind limb grip strength of rats and mice. The apparatus consists of an adjustable trough and a push-pull strain gauge with a triangular brass ring which is grasped by the animal with its forelimbs. The animal is pulled on the tail until the grip is broken. The animal continues to be pulled along the trough until the hind limbs grasp a T-shaped bar being also attached to push-pull strain gauge. The trial is completed when the grip of the hind limbs is also broken. Fore- and hind limb strength are measured. Dose-response curves could be established with various doses of chlordiazepoxide and phenobarbital.

Barcley et al. (1981) described the tight rope test for testing performance in mice.

Simiand et al. (1989) used a test originally described by Fleury (1957). A mouse held by the tail is placed on a small metallic grid which the animal gripped with its forepaws. The grid is than loaded with weights until the mouse could not longer support the weight of the grid. The endpoint is the maximal weight supported by the animal at least for 2 s. ED_{50}-values can be calculated for various centrally active skeletal muscle relaxants such as the benzodiazepines.

Deacon and Gardner (1984) described the pull-up test in rats. A rat is held by its hind legs in an inverted position. The time taken by the rat to pull itself up and grasp the hand of the experimenter is used as the test parameter.

Novack and Zwolshen (1983) tested muscle relaxants in various models, such as morphine-induced rigidity in rats, decerebrate rigidity in cats and the polysynaptic linguomandibular reflex in cats.

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E.1.3.4 Rotarod method

PURPOSE AND RATIONALE

The test is used to evaluate the activity of drugs interfering with motor coordination. In 1956, Dunham and Miya suggested that the skeletal muscle relaxation induced by a test compound could be evaluated by testing the ability of mice or rats to remain on a revolving rod. This forced motor activity has subsequently been used by many investigators. The dose which impairs the ability of 50% of the mice to remain on the revolving rod is considered the endpoint.

PROCEDURE

The apparatus consists of a horizontal wooden rod or metal rod coated with rubber with 3 cm diameter attached to a motor with the speed adjusted to 2 rotations per minute. The rod is 75 cm in length and is divided into 6 sections by plastic discs, thereby allowing the simultaneous testing of 6 mice. The rod is in a height of about 50 cm above the table top in order to discourage the animals from jumping off the roller. Cages below the sections serve to restrict the movements of the animals when they fall from the roller. Male mice (CD-1 Charles River strain) with an weight between 20 and 30 g undergo a pretest on the apparatus. Only those animals which have demonstrated their ability to remain on the revolving rod for at least 1 minute are used for the test. The test compounds are administered intraperitoneally or orally. Thirty minutes after intraperitoneal or 60 min after oral administration the mice are placed for 1 min on the rotating rod. The number of animals falling from the roller during this time is counted.

Using different doses, ED_{50} values can be calculated. Moreover, testing at various time intervals, time-response curves can be obtained.

CALCULATION

Percent animals falling from the rotarod within the test period is calculated for every drug concentration tested. ED_{50} is defined as the dose of drug at which 50% of the test animals fall from the rotarod.

CRITICAL ASSESSMENT OF THE TEST

Many central depressive drugs are active in this test. Benzodiazepines, such as diazepam and flurazepam, have ED_{50} values below 1 mg/kg i.p. The activity of neuroleptics, such as chlorpromazine or haloperidol, is in the same range. In this way, the test does not really differentiate between anxiolytics and neuroleptics but can evaluate the muscle relaxant potency in a series of compounds such as the benzodiazepines. Moreover, the test has been used in toxicology for testing neurotoxicity.

MODIFICATIONS OF THE METHOD

A comparison of the rotarod method in rats with other tests, such as blockade of morphine-induced rigidity in rats, decerebrate rigidity in cats, and polysynaptic-monosynaptic reflex preparations in cats was published by Novack and Zwolshen (1983).

Rozas et al. (1997) described a drug-free rotarod test that was used to evaluate the effects of unilateral 6-hydroxydopamine lesions, nigral grafts, and subrotational doses of apomorphine. The rotarod unit was automated and interfaced with a personal computer allowing automatic recording of the time that each rat was able to stay on the rod at different rotational speeds.

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E.1.3.5 Influence on polysynaptic reflexes

PURPOSE AND RATIONALE

Inhibition of polysynaptic reflexes is considered to be the major mode of action of muscle relaxants. Polysynaptic transmission can be measured by the flexor reflex of the hindpaw in anesthetized rats, whereas the monosynaptic Hoffmann reflex is measured by electromyographic recordings from the plantar foot muscle (Block and Schwarz 1994; Schwarz et al. 1994, 1996).

PROCEDURE

Male Wistar rats (250–280 g) are anesthetized with urethane (400 mg/kg i.p.) and α-chloralose (80 mg/kg i.p.). For the flexor reflex a hindpaw is stimulated with a pair of fine subcutaneous needle electrodes (5 square-wave shocks at 500 Hz, 0.2 ms duration and 3.0 reflex threshold). Electromyographic recordings are made with a pair of fine needle electrodes inserted into the ipsilateral tibialis muscle. Seven consecutive electromyographic recordings are amplified, and band-pass filtered (8–10 Hz), collected at a sample rate of 10 Hz, averaged and evaluated using a Signal Averager (CED, Cambridge, UK) on an IBM-compatible personal computer. Ten consecutive responses are averaged before and 20 min after i.p. application of drug.

For stimulation of the Hoffmann reflex a pair of needle electrodes is transcutaneously inserted into the surrounding of the tibial nerve (single square-wave shocks, 0.2 ms duration at 2.0 threshold). Electromyographic recordings are made with a pair of skin clip surface electrodes from the plantar foot muscle. Low intensity electrical stimulation of the tibial nerve elicits a reflex response similar to the human Hoffmann (H) reflex, which has been attributed to monosynaptic excitation of spinal α-motoneurons predominantly by primary muscle spindle afferent fibers. With increasing stimulus strength, the H-wave is preceded by an electromyographic wave, the M wave, which is due to a direct excitation of axons of α-motoneurons.

In all reflex experiments, values measured after solvent or drug application are expressed as a percentage of the corresponding pre-injection value.

EVALUATION

Statistical evaluation of group differences is performed using the Mann-Whitney U-test. Statistical analysis for dose dependency of drug effects is carried out by the Kruskal-Wallis test.

MODIFICATIONS OF THE METHOD

Ono et al. (1990), Farkas and Ono (1995), Hasegawa and Ono (1996), Otsu et al. (1998) recorded spinal reflexes from spinalized and non-spinalized rats anesthetized with α-chloralose and urethane. Laminectomy was performed in the lumbo-sacral region. Ventral and dorsal roots of the segments L4 and L5 were isolated. A skin pouch was formed at the site of the dissection to cover the exposed tissues with liquid paraffin kept at 36 °C. The dorsal root of L5 was placed on bipolar silver wire electrodes for stimulation (0.2 Hz, 0.05 ms, supramaximal). The ipsilateral ventral root of L5 and the dorsal root of L4 were placed on bipolar wire electrodes for recording. Monosynaptic and polysynaptic reflexes and dorsal root-dorsal root reflexes were evoked in the L5 ventral root and in the L4 dorsal root, respectively. These reflex potentials were amplified, displayed on an oscilloscope and averaged 8 times by an averaging computer.

Turski and Stephens (1993) recorded monosynaptic Hoffman reflexes in NMRI **mice** anesthetized with 80 mg/kg α-chloralose i.p. +400 mg/kg urethane i.p. The tibial nerve was stimulated with single square-wave pulses, 0.2 ms duration at 1.2–1.6 times the nerve threshold. Electromyogram recordings were made with a pair of skin clip surface electrodes from the plantar foot muscle. For recording polysynaptic flexor reflexes, the tibial nerve was stimulated with five square-wave pulses at 500 Hz, 0.2 ms duration at 3.0 times the nerve threshold. Electromyogram recordings were made with a pair of wire electrodes inserted percuteneously into the ipsilateral tibial muscle.

Furthermore, these authors used **genetically spastic rats**. A mutant strain of Wistar rats, which carries an autosomal recessive gene defect, is characterized by a progressive paresis of the hindlimbs with increased tone on the extensor muscles (Pittermann et al. 1976). This genetically determined syndrome of spasticity in the rat permits quantitative evaluation of the effect of drugs on muscle tone by recording activity in the electromyogram from a hindlimb extensor muscle (Klockgether et al. 1985; Turski et al. (1990).

Farkas et al. (1989), Tarnava et al. (1989) studied the effects of drugs on the reflex potentials evoked by afferent nerve stimulation and recorded from the spinal roots in unanesthetized spinal **cats**. An analog integrating method was used for quantitative evaluation of the reflex potentials. The amplified and band-pass filtered signals from the ventral root (monosynaptic reflex and polysynaptic reflex) and from the dorsal root (dorsal root reflex and dorsal root potential) were fed into signal-selectors, which transmitted the input signals only within the chosen post-stimulus intervals. Thus, the various components of the reflex potentials were separated according to their latencies.

Shakitama et al. (1997) recorded ventral root reflex potential in anesthetized rats and ventral and dorsal root potentials in anesthetized intact and spinalized cats.

Suzuki et al. (1995) studied the recovery of reflex potentials after spinal cord ischemia produced by occlusion of the thoracic aorta and the bilateral internal mammary arteries for 10 min in cats.

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E.1.3.6 Masticatory muscle reflexes

PURPOSE AND RATIONALE

The temporomandibular joint dysfunction syndrome, characterized by pain and clicking in the temporomandibular joint and limitation of function, involves a condition caused by hypertonia combined with parafunctional habits, such as clenching or grinding of teeth and hyperreflexia (Laskin and Block 1986) which may be treated by muscle relaxants. Ozawa et al. (1996) studied the effects of a centrally acting muscle relaxant on *masticatory* muscle reflexes in rats. Both monosynaptic and polysynaptic reflexes can be studied using this model.

PROCEDURE

For recording the **monosynaptic tonic vibration reflex** of the masseter muscle, Wistar rats are anesthetized with ether, intubated with a tracheal cannula and fixed into a stereotactic apparatus. Decerebration is performed by radiofrequency lesion of the midbrain using a Lesion Generator (Radionics, RFG-4, USA) and a lesioning electrode inserted into the midbrain. After lesioning, ether anesthesia is discontinued. The tonic vibration reflex of the masseter muscle, recorded as electromyogram, is induced every 10–12 s by a sinusoidal vibration (100–500 Hz, 2 s) which is applied to the mandi-bula, and is delivered by a vibration generator driven by a low frequency oscillator and an amplifier. The evoked electromyogram is amplified by a biophysical amplifier and recorded on a thermal array recorder. The root mean square of the electromyogram is also recorded through an integrator.

For recording the **polysynaptic jaw opening reflex**, the animals are anesthetized by intraperitoneal pentobarbital-Na (50 mg/kg), fixed into position on their back and intubated with a tracheal cannula. Intrapulpal stimulation (0.5 Hz, 0.2 s in pulse duration, supramaximal intensity) delivered by an electrical stimulator is performed via a dental reamer inserted into the dental pulp of the mandibula. The jaw opening reflex recorded as phasic component of the electromyogram evoked in the ipsilateral digastric muscle is amplified by a biophysical amplifier and recorded on a thermal array recorder.

For recording of the **polysynaptic tonic periodontal masseteric reflex** (Funakoshi and Amano 1974) the animals are anesthetized by intraperitoneal injection of pentobarbital-Na (35 mg/kg), which is supplemented as required, intubated with a tracheal cannula and fixed onto a stereotaxic apparatus. The maxillary incisor is stimulated by pressing for 5 s every 5 min using a vibration generator driven by a trapezoid generator. The electromyogram responses to this stimulation are amplified by a biophysical amplifier and recorded on a thermal array recorder. The evoked electromyogram is transformed into square-wave pulses, fed into a staircase generator and recorded on a thermal array recorder.

EVALUATION

The significance of differences between the control and the drug-treated groups is evaluated with Dunnett's test.

MODIFICATIONS OF THE METHOD

Boucher et al. (1993) performed microinfusions of excitatory amino acid antagonists into the trigeminal sensory complex of freely moving rats while record-

ing the long latency jaw opening reflex elicited by electrical stimulation of the dental pulp.

Bakke et al. (1998) studied in anesthetized rats neurokinin receptor mechanisms in the increased jaw muscle activity which can be evoked by injection of the small fiber excitant and inflammatory irritant mustard oil into the temporomandibular joint region.

Alia et al. (1998) preformed intra-oral administration of a NK_1 antagonist in freely moving guinea pigs during recording the short- (6–10 ms) and long-latency (18–26 ms) jaw-opening reflex elicited by electrical stimulation of the lower incisor tooth pulp.

Huopaniemi et al. (1988) determined the threshold of the tooth-pulp evoked jaw-opening reflex after naloxone in barbiturate-anesthetized cats.

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E.2 Tests for anxiolytic activity

E.2.0.1 General considerations

Definitions in psychopharmacology have been coined by the activity of special compounds or chemical classes found in patients. This is not only true for the term "neuroleptic" but also for the term "anxiolytic". Other terms have been "ataractic" or "psycholeptic". Anxiolytics are derived from "tranquilizers", such as meprobamate which was used widely until the advent of benzodiazepines. The property which these drugs have in common is the alleviation of anxiety, thus explaining the term "anxiolytic". These agents are used for the relatively minor disorders of the nonpsychotic or neurotic type, whereas the antipsychotic agents (phenothiazines, butyrophenones) are given mainly to combat the more severe psychotic or schizophrenic reactions. Thus, the terms anti-anxiety and antipsychotic indicate a qualitative distinction in the clinical use and mode of action of the drug. Pathological anxiety in man has been defined by its interference with normal functions, by manifestations of somatic disorders, emotional discomfort, interference with productivity at work, etc. This complex characterization of anxiety in man already indicates the difficulties to find appropriate pharmacological models. Therefore, several tests have to be performed to find a spectrum of activities which can be considered to be predictive for therapeutic efficacy in patients. For *in vivo* studies, most investigators use a battery of anticonvulsive tests, anti-aggressive tests and evaluation of conditioned behavior.

Most of the actions of benzodiazepines are thought to be mediated by potentiation of g-amino-butyric acid (GABA). Two subtypes of GABA receptors ($GABA_A$ and $GABA_B$) have been described. Moreover, specific binding sites for benzodiazepines have been discovered near these GABA receptors in various areas of the brain. These sites occur in a macromolecular complex that includes GABA-receptors, benzodiazepine receptors and receptors for other drugs, and a chloride channel. The benzodiazepines potentiate the neurophysiological actions of GABA at the chloride ion channel by increasing the binding of GABA to $GABA_A$ receptors. This implies that the $GABA_A$ receptor is involved in anxiety and that its direct activation would have an anxiolytic effect. Based in these findings various *in vitro* tests have been developed.

More recently, research has focused on the therapeutic potential of blocking excitatory amino acids – in particular glutamate. Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA) and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987). Some methods are described in Sect. E.3 Anti-epileptic activity.

Serotonin may play a role in anxiety, since treatment with drugs that reduce serotoninergic function, including benzodiazepines, have anxiolytic effects in animal models (Dourish et al. 1986). Several subtypes of serotonin receptors have been elucidated, e.g. $5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$. $5\text{-}HT_{1C}$, $5\text{-}HT_{1D}$, $5\text{-}HT_2$, $5\text{-}HT_3$. Further differentiation is underway. $5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$, and $5\text{-}HT_3$ receptors are considered to be involved in the effect of anti-anxiety and novel antipsychotic drugs (Peroutka 1988; Costall et al. 1988). Some *in vitro* methods are described in Sect. E.6 Antidepressant activity and Sect. E.5 Neuroleptic activity.

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E.2.1 *In vitro* methods

E.2.1.1 *In vitro* assay for GABAergic compounds: [^{3}H]-GABA receptor binding

PURPOSE AND RATIONALE

GABA is known to be an important inhibitory neurotransmitter in the brain. Abnormalities in the GABA-system have been found in neurological and psychiatric diseases such as Huntingdon's chorea, anxiety, panic attacks, schizophrenia and epilepsy. GABA is also implicated in the mechanism of benzodiazepines and related CNS-drugs. Radiolabeled GABA is bound to synaptic membrane preparations of mammalian brain. The labeling of the synaptic receptor with ^{3}H-GABA requires careful attention to possible interference from nonsynaptic binding since ^{3}H-GABA can also bind nonspecifically to plasma membranes. The most prominent of which is the sodium dependent binding of GABA to brain membranes, a process which appears to be associated with the transport (uptake) sites of GABA. Sodium-independent binding of ^{3}H-GABA has characteristics consistent with the labeling of GABA receptors. In addition, the relative potencies of several amino acids in competing for these binding sites parallel their abilities to mimic GABA neurophysiologically. Therefore, the sodium-independent binding of ^{3}H-GABA provides a simple and sensitive method to evaluate compounds for GABA-mimetic properties.

GABA receptors have been divided into $GABA_A$ and $GABA_B$ receptor subtypes which by themselves form receptor families (Matsumoto 1989; Knott and Bowery 1991; Möhler 1992).

PROCEDURE

Reagents

- 0.05 M Tris-maleate buffer (pH 7.1)
- 6.05 g of Tris-base are dissolved in distilled water and made up to 1 000 ml. 5.93 g of Tris- maleate are dissolved in 500 ml of water. The 0.05 M Tris maleate, pH 7.1 buffer is prepared by slowly adding Tris maleate to the Tris-base solution until the pH reaches 7.1.
- 0.32 M Sucrose: 109.5 g of sucrose are dissolved in distilled water and filled up to 1 000 ml. The solution is stored at 4 °C.
- ^{3}H-GABA (specific activity approximately 40 Ci/mmol) is made up to a concentration of 780 nmol in distilled water and 20 µl is added to each test tube (yielding a final concentration of 15 nmol in the assay). Isoguvacine or muscimol is prepared by dissolving 8.35 mg of isoguvacine or 6.40 mg of muscimol in 10 ml water. Twenty µl of these solutions when added to 1 ml of incubation medium give a final concentration of 0.1 mM isoguvacine or muscimol.
- Test drugs: 1 mM stock solutions are initially prepared. These are serially diluted to the required concentrations prior to the addition to the incubation mixture. Final concentrations are usually from 2×10^{-8} to 1×10^{-5} M.

Tissue preparation

Male Charles-River rats (100–150 g) are decapitated and their whole brains rapidly removed and homogenized in 15 vol of ice-cold 0.32 M sucrose. The homogenate is centrifuged at 1000 *g* for 10 min. The pellet (nuclear fraction) is discarded and the supernatant fluid is recentrifuged at 20 000 *g* for 20 min. The supernatant is discarded and the crude mitochondrial pellet is resuspended in 15 vol distilled water using a Tekmar homogenizer. The suspension is centrifuged at 8 000 *g* for 20 min. The supernatant is collected and used to carefully resuspend, using a gentle squirling motion, the pellet's soft, upper, buffy layer. This sus-

pension is then centrifuged at 48 000 *g* for 20 min. The final crude synaptic membrane pellets are resuspended (without homogenization) in 15 volumes of distilled water and centrifuged at 48 000 *g* for 20 min. The supernatant is discarded, and the centrifuge tubes containing the pelleted membranes are capped with parafilm and stored frozen at –70 °C.

Assay procedure

A frozen membrane pellet from one whole rat brain is resuspended in 15 volumes of 0.05 M Tris-maleate buffer (pH 7.1) by homogenization at 4 °C. Triton X-100 is added to a final concentration of 0.05%. This suspension is then incubated at 37 °C for 30 min followed by centrifugation at 48 000 *g* for 10 min. The supernatant is discarded and the pellet resuspended by homogenization in the same volume of 0.05 Tris-maleate buffer (pH 7.1) at 4 °C. The preincubation with Triton enhances specific GABA receptor binding while lowering non-specific binding.

For the standard Na-independent ^{3}H-GABA binding assay procedure, aliquots of the previously frozen, Triton treated crude synaptic membranes are incubated in triplicate at 4 °C for 5 min in 0.05 M Tris-maleate buffer (pH 7.1) containing 15 nM ^{3}H-GABA alone or in the presence of 0.1 mM isoguvacine or muscimol, or the test drug.

The procedure is as follows:

- 1 ml of the 0.05 M Tris-maleate homogenate
- 20 µl of ^{3}H-GABA
- 20 µl of test drug or 20 ml of 0.1 mM isoguvacine or muscimol.

After incubation at 4 °C for 5 min, the reaction is terminated by centrifugation for 15 min at 5 000 rpm. The supernatant fluid is aspirated and the pellet washed twice with 1 ml of the Tris-maleate buffer. Two ml of liquiscint are added to each tube which is then vigorously vortexed. The contents of the tubes are transferred to scintillation vials, and the tubes rinsed with an additional 2 ml of cocktail. An additional 6 ml of liquiscint are added to each scintillation vial. The radioactivity is measured by liquid scintillation photometry.

EVALUATION

Specific ^{3}H-GABA binding is defined as the radioactivity which can be displaced by a high concentration of unlabeled GABA and is obtained by subtracting from the total bound radioactivity the amount of radioactivity bound in the presence of 0.1 mM isoguvacine. Results are converted to percent of specifically bound ^{3}H-GABA displaced by a given concentration of test drug. IC_{50} values with 95% confidence limits are then obtained by computer derived linear regression analysis.

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E.2.1.2 $GABA_A$ receptor binding

PURPOSE AND RATIONALE

Two subtypes of GABA receptors have been identified:

1. $GABA_A$ receptor for which muscimol is the typical agonist, whereas bicucculine, picrotoxin, and SR 95 531 are antagonists, and
2. $GABA_B$ receptor, for which baclofen is the typical agonist.

The $GABA_A$ receptor directly gates a Cl^- ionophore and has modulatory binding sites for benzodiazepines, barbiturates, neurosteroids and ethanol. By contrast, $GABA_B$ receptors couple to Ca^{2+} and K^+ channels via G proteins and second messenger systems; they are activated by baclofen and are resistant to drugs that modulate $GABA_A$ receptors.

Several subtypes of $GABA_A$ receptors have been identified by ligand binding studies (Kleingoor et al. 1991; Turner et al. 1992; Gusti et al. 1993). Molecular biology techniques revealed the $GABA_A$ receptor to be assembled as a pentameric structure from different subunit (α, β, γ and δ subunit) families making it possible that a very large number of such heteromeric $GABA_A$ receptors exist in the mammalian central and peripheral nervous system (Krogsgaard-Larsen et al.

1994; Lambert et al. 1995; Smith and Olsen 1995; Costa 1998). A total of 19 genes encoding $GABA_A$ receptor subunits are known, while several additional isoforms can occur as splicing variants of some of these (Barnard 1998). Probably more than 500 distinct $GABA_A$ receptor subtypes exist in the brain (Sieghart 2000). Sequences of six α, three β, three γ, one δ, three ρ, one ε, one π and one θ $GABA_A$ receptor subunits have been reported in mammals (Barnard 2000; Alexander et al. 2001). More insight into the pharmacological functions of $GABA_A$ receptor subtypes is expected from studies in gene-knockout mice and by knock-in point mutations (Rudolph et al. 2001).

$GABA_C$ receptors were described as a pharmacologically distinct group by Bormann and Feigenspan (1995), Johnston (1996), Bormann (2000), Zhang et al. (2001). These receptors are Cl^- pores that are insensitive to both bicucculine and baclofen. An IUPHAR Committee has recommended in 1998, that the term $GABA_C$ should be avoided and classified bicucculine- and baclofen-insensitive GABA receptors as a minor subspecies of $GABA_A$ receptors of the 'AO' type.

In rat brain membranes, only the $GABA_A$ receptor is labeled with the $GABA_A$ selective radioligand ^{3}H-SR 95 531 in the given concentration range. The assay allows specifically the estimation of the test drug's binding characteristics to the $GABA_A$ receptor subpopulation.

PROCEDURE

Materials

Radioligand: ^{3}H-SR 95 531 (^{3}H-2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)-pyridazinium bromide (New England Nuclear, Boston)

Membrane preparation

Rats are killed by decapitation, the brains are quickly dissected and after separation from the cerebellum placed into ice-cold sucrose solution. The brains (approximately 20 g wet weight) are then homogenized in a glass Teflon potter (1 g brain weight/15 ml 320 mM D(+)-sucrose solution), and centrifuged at 1 000 *g* at 4 °C for 10 min. The pellets are discarded and the supernatants centrifuged at 20 000 *g* for 20 min. The resulting supernatants are discarded and the pellets are lysed by hypoosmotic shock (addition of 20 volumes of ice-cold bidistilled water). After homogenization in a glass Teflon homogenizer, the suspension is stirred under cooling for 20 min, and centrifuged at 48 000 *g* for 20 min. The resulting pellets are resuspended in ice-cold bidistilled water, the suspension is stirred and recentrifuged as before. The final pellets are resuspended in the incubation buffer (50 mM Tris-HCl and 100 mM $MgCl_2 \times 6\ H_2O$, pH 7.4) corresponding to 1 g brain wet weight/1 ml buffer. The membrane suspension is immediately stored in aliquots of 1 ml at –20 °C. Protein content of the membrane suspension is determined according to the method of Lowry et al. (1951) with bovine serum albumin as standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed and diluted 1 : 20 with bidistilled water. After stirring for 10 min, the membrane suspension is centrifuged at 50 000 *g* for 10 min. The resulting pellets are resuspended in ice-cold incubation buffer, yielding a membrane suspension with a protein content of 1 mg/ml.

Assay

For each concentration, assays are performed in triplicate. The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation experiments

Total binding:

- 50 ml ^{3}H-SR 95531 (12 concentrations, 2×10^{-9} to 1×10^{-7} M)
- 50 ml incubation buffer

Non-specific binding:

- 50 ml ^{3}H-SR 95531 (4 concentrations, 2×10^{-9} to 1×10^{-7} M)
- 50 ml (+) bicucculine (10^{-4} M)

Competition experiments

- 50 ml ^{3}H-SR 95531 (1 constant concentration, $8–10 \times 10^{-9}$ M)
- 50 ml incubation buffer without or with non-labeled test drug (15 concentrations, 10^{-10} to 10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample. The samples are incubated for 30 min at 4 °C. The reaction is stopped by subjecting the total incubation volume to rapid vacuum filtration over glass fiber filters. Thereby, the membrane-bound radioactivity is separated from the free radioactivity. Filters are washed immediately with approximately 20 ml ice-cold rinse buffer (50 mM Tris HCl, pH 7.4) per sample. The membrane-bound radioactivity is measured after addition of 2 ml liquid scintillation cocktail per sample in a Packard liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding – non-specific binding.

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-SR 95 531 versus non-labeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D{}^3\mathrm{H} \times IC_{50}}{K_D{}^3\mathrm{H} + [^3\mathrm{H}]}$$

IC_{50} = concentration of the test drug, which inhibits 50% of specifically bound ^{3}H-SR 95 531 in the competition experiment.
$[^3\mathrm{H}]$ = concentration of ^{3}H-SR 95 531 in the competition experiment.
$K_D{}^3\mathrm{H}$= dissociation constant of ^{3}H-SR 95 531, determined from the saturation experiment.

The K_i-value of the test drug is that concentration, at which 50% of the receptors are occupied by the test drug.

MODIFICATIONS OF THE METHOD

Binding to the agonist site of the $GABA_A$ receptor can be measured with [^{3}H]muscimol (Snodgrass 1978; Williams and Risley 1979; Martini et al. 1983).

A membrane fraction of whole brains (except cerebellum) from male Wistar rats is prepared by standard techniques. Ten mg of membrane preparation is incubated with 1 nM [^{3}H]muscimol for 10 min at 0 °C. Non-specific binding is estimated in the presence of 100 nM muscimol. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [^{3}H]muscimol.

The $GABA_A$ receptor chloride channel can be studied by binding with [^{3}H]t-butylbicycloorthobenzoate ([^{3}H]TBOB) (Schwartz and Mindlin 1988; Lewin et al. 1989).

A membrane fraction of whole brains (except cerebellum) from male Wistar rats is prepared by standard techniques. 0.4 mg of membrane preparation is incubated with 3 nM [^{3}H]TBOB for 15 min at 15 °C. Non-specific binding is estimated in the presence of 200 µM picrotoxin. Membranes are filtered and washed 3 times and the filters are counted to determine [^{3}H]TBOB specifically bound.

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E.2.1.3 $GABA_B$ receptor binding

PURPOSE AND RATIONALE

Baclofen, as an analogue of the inhibitory neurotransmitter γ-aminobutyric acid (GABA), binds as agonist to the subtype B of the GABA receptor. Baclofen is effective in the treatment of spasticity caused by multiple sclerosis or other diseases of the spinal cord, particularly traumatic lesions. Studies of similar compounds may lead to other effective antispasmodic drugs.

In rat cerebellar membranes, only the $GABA_B$ receptor is labeled in the given concentration range of the $GABA_B$ selective agonist ^{3}H-(–)baclofen. The assay allows specifically the estimation of the test drug's binding characteristics at the $GABA_B$ subtype receptor population.

The receptor on which baclofen acts is coupled via G_i/G_o proteins to Ca^{2+} and K^+ channels as well as adenyl cyclase in neurons and hence is classified as a metabotropic receptor.

As in other receptor families, heterogeneity of the $GABA_B$ receptor has been found (Scherer et al. 1988; Bittiger et al. 1992; Bonanno and Raiteri 1992, 1993a,b; Bowery 1993; Lanza et al. 1993). At least 3 distinct subtypes have be identified:

a) the postsynaptic receptor linked via a G-protein to a K^+ channel which upon stimulation by GABA hyperpolarizes the neuron,
b) the presynaptic autoreceptor at GABA nerve endings; blockade of this receptor augments the release of GABA in electrically stimulated rat cortical slices,
c) the presynaptic heteroreceptor at glutamate nerve endings; blockade with $GABA_B$ antagonists increases release of glutamate from K^+ stimulated cortical slices.

The structure of $GABA_B$ receptors was identified when isoforms were detected (Kaupmann et al. 1997). Potent $GABA_B$ antagonists were described (Bittiger et al. 1992, 1993; Froestl et al. 1996).

PROCEDURE

Materials

Radioligand: ^{3}H-(–)baclofen, specific activity 1.11–1.85 TBq/mmol (30–50 Ci/Mmol) New England Nuclear, Boston

Membrane preparation

Rats are killed by decapitation, the cerebella quickly removed, and placed into ice-cold preparation buffer (50 mM Tris-HCl, pH 7.4). Approximately 5 g wet weight of the cerebella are homogenized using a glass Teflon potter, corresponding to 1 g cerebellum wet weight/50 ml buffer, and centrifuged at 48 000 *g* at 4 °C for 10 min. The pellets are resuspended in approximately 270 ml preparation buffer, and centrifuged as before. The final pellets are dissolved in preparation buffer, corresponding to 1 g cerebellum wet weight/30 ml buffer. The membrane suspension is immediately stored in aliquots of 5–10 ml at –77 °C. Protein content of the membrane suspension is determined according to the method of Lowry et al. with bovine serum albumin as a standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed, and centrifuged at 50 000 *g* for 20 min. The pellets are resuspended in the same volume of incubation buffer, and stirred for 45 min at room temperature. The suspension is recentrifuged as before. This washing step is repeated 3 times. The resulting pellets are resuspended in ice-cold incubation buffer in a volume, yielding a membrane suspension with a protein content of 1 mg/ml. The membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Assay

For each concentration samples are used in triplicate. The total volume of each incubation sample is 200 µl (microtiter plates). The concentration of ^{3}H-(–)baclofen is constant in all samples ($1.8–2 \times 10^{-8}$ M).

Saturation experiments
Total binding:
- 50 µl ^{3}H-(–)baclofen
- 50 µl non-radioactive racemic baclofen (15 concentrations, 0–1.2×10^{-6} M)

Non-specific binding:
The measurement of the non-specific binding is performed at the lowest concentration of the saturation range, i.e. 1.8×10^{-8} M of the ^{3}H-(–)baclofen without non-radioactive racemic baclofen.

- 50 µl ^{3}H-(–)baclofen
- 50 µl gamma-aminobutyric acid.

Competition experiments
- 50 µl ^{3}H-(–)baclofen
- 50 µl incubation buffer without or with labeled test drug (15 concentrations, 10^{-10}–10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (1 mg protein/1 ml). The samples are incubated for 60 min at 4 °C. The reaction is stopped by subjecting the total incubation volume to rapid vacuum filtration over glass fiber filters. Thereby, the membrane bound is separated from the free radioactivity. The filters are washed with approximately 20 ml ice-cold buffer. The retained membrane bound radioactivity on the filter is measured after addition of 0.3 ml ethylene glycol monomethyl ether and 2 ml liquid scintillation cocktail per sample and an equilibration time of 1 h in a Packard liquid scintillation counter.

EVALUATION
The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding – non-specific binding.

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-(–)baclofen versus non-labeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D{}^3\mathrm{H} \times IC_{50}}{K_D{}^3\mathrm{H} + [^3\mathrm{H}]}$$

IC_{50} = concentration of the test drug, which inhibits 50% of specifically bound ^{3}H-(–)baclofen in the competition experiment.
$[^3\mathrm{H}]$ = concentration of ^{3}H-(–)baclofen in the competition experiment.
$K_D{}^3\mathrm{H}$ = dissociation constant of ^{3}H-(–)baclofen, determined from the saturation experiment.

The K_i-value of the test drug is the concentration, at which 50% of the receptors are occupied by the test drug.

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CRITICAL ASSESSMENT OF GABA BINDING TESTS

Without any doubt, GABA is the most important inhibitory transmitter in the central nervous system. One may expect important new developments by attempts to influence the GABAergic system specifically by synthetic compounds. In general, $GABA_A$ receptor agonists are central nervous system depressants, muscle relaxants, and possess some nociceptive properties, whereas the receptor antagonists are convulsants. Most benzodiazepine binding sites seem to be associated with $GABA_A$ receptors combined with a chloride channel. Among $GABA_B$ receptor agonists, only baclofen has achieved success in clinical use. Results of ongoing research with receptor subtypes will result in the development of new therapeutic agents.

E.2.1.4 Benzodiazepine receptor: [^{3}H]-flunitrazepam binding assay

PURPOSE AND RATIONALE

Experiments using ^{3}H-diazepam or ^{3}H-flunitrazepam have demonstrated specific binding sites in CNS membrane preparations that satisfy the criteria for pharmacological receptors, e.g. saturability, reversibility, stereoselectivity and significant correlation with *in vivo* activities of the drugs in this class.

Heterogeneity of benzodiazepine receptors has been reported (Klepner et al. 1979; Supavilai and Karobath 1980; Hafely et al. 1993; Davies et al. 1994). There are four classes of benzodiazepine and non-benzodiazepine high affinity ligands for benzodiazepine recognition sites associated with $GABA_A$ receptors: The first class (e.g. diazepam, flunitrazepam, alprazolam) facilitates the action of GABA, increasing the opening frequency of Cl^- channels. These ligands are called full positive allosteric modulators, or full agonists. A second class of ligands, which includes the β-carbolines, can decrease the opening frequency of Cl^- channels. These ligands are known as full negative allosteric modulators, or full inverse agonists. A third class (e.g. flumazenil) binds with high affinity to benzodiazepine recognition sites, but it can also prevent the GABA modulations elicited by positive or negative allosteric modulators; this class is called a modulator antagonist. A fourth class of ligands for benzodiazepine recognition sites is known to elicit either partial amplification or partial attenuation of GABA action at various $GABA_A$ receptors, and comprises the class called partial positive and partial negative allosteric modulators or partial agonists and partial inverse agonists, respectively.

The names **ω_1, ω_2, and ω_3-receptor subtypes** have been proposed to replace the nomenclature of benzodiazepine BZ_1, BZ_2, and BZ_p receptors (Langer and Arbilla 1988; Langer et al. 1990; Griebel et al. 1999a,b).

PROCEDURE

Reagents

- [Methyl-^{3}H]-Flunitrazepam (70–90 Ci/mmol) can be obtained from New England Nuclear.
- Clonazepam HCl can be obtained from Hoffmann La Roche

Tissue preparation

Male Wistar rats are decapitated and the brains rapidly removed. The cerebral cortices are removed, weighed and homogenized with a Potter-Elvejhem homogenizer in 20 volumes of ice-cold 0.32 M sucrose. This homogenate is centrifuged at 1 000 *g* for 10 min. the pellet is discarded and the supernatant is centrifuged at 30 000 *g* for 20 min. The resulting membrane pellet is resuspended in 40 volumes of 0.05 M Tris buffer, pH 6.9.

Assay

1 ml 0.05 Tris buffer, pH 6.9
560 µl H_2O

70 µl 0.5 M Tris buffer, pH 6.9
50 µl ^{3}H-Flunitrazepam
20 µl vehicle (for total binding) or 0.1 mM Clonazepam (for non-specific binding) or appropriate drug concentrations.
300 µl tissue suspension.

The tubes containing ^{3}H-flunitrazepam, buffer, drugs and H_2O are incubated at 0–4 °C in an ice bath. A 300 µl aliquot of the tissue suspension is added to the tubes at 10-s intervals. The timer is started with the addition of the mixture to the first tube. The tubes are then incubated at 0–4 °C for 20 min and the assay stopped by vacuum filtration through Whatman GF/B filters. This step is performed at 10-s intervals. Each filter is immediately rinsed with three 5-ml washes of ice-cold buffer, pH 6.9. The filters are counted in 10 ml of liquid scintillation counting cocktail.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of clonazepam. Specific binding is approximately 97% of total ligand binding. The percent inhibition at each drug concentration is the mean of triplicate determinations. IC_{50} calculations are performed using log-probit analyses.

CRITICAL ASSESSMENT OF THE METHOD

Binding to the benzodiazepine receptor is not absolutely predictive for anxiolytic activity. A range of compounds have been discovered that do not have the benzodiazepine structure but that do interact with the benzodiazepine receptors (Gardner 1988; Byrnes et al. 1992). They may have a different pharmacological profile *in vivo*.

MODIFICATION OF THE METHOD

Takeuchi et al. (1992) developed a non-isotopic receptor assay for benzodiazepine drugs using the biotin-1 012-S conjugate. The free conjugate in the supernatant was determined with a solid-phase avidin-biotin binding assay.

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E.2.1.5 Serotonin receptor binding

E.2.1.5.1 General considerations

Several surveys on 5-HT receptors and their classification have been published, e.g., by Humphrey et al. (1993), Peroutka (1993), Boess and Martin (1994), Hoyer et al. (1994), Kebabian and Neumeyer (1994), Martin and Humphrey (1994), Saxena (1994), Brancheck (1995), Sleight et al. (1995), Bockaert et al. (1997), Branchek and Zgombick (1997), Briley et al. (1997), Costal and Naylor (1997), Glennon and Dukat (1997), Göthert and Schlicker (1997), Hamon (1997), Hartig (1997), Hoyer and Martin (1997), Jacobs and Fornal (1997), Roth and Hyde (1997), Uphouse (1997), Martin (1998), Martin and Eglen (1998), Saxena et al. (1998).

The classification has evolved from a scheme recognizing three classes (5-HT_{1like}, 5-HT_2 and 5-$HT_{3)}$ to one accepted by the NC-IUPHAR subcommittee for 5-hydroxytryptamine (serotonin) receptors in which seven classes embrace 14 distinct receptor subtypes (Martin 1988; Martin and Eglen 1998). Some revisions of the nomenclature were made:

Renaming the 5-HT_{1C} receptor to 5-HT_{2C} on the basis of recognitory, transductional and structural identity with the 5-HT_2 family.

Alignment of the classification scheme with the human genome, meaning that human receptors are given pre-eminence in the nomenclature.

Renaming the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ subtypes to 5-HT_{1D} and 5-HT_{1B}, respectively.

Recognition that the '5-HT_{1like}' positively coupled to adenylate cyclase and mediating smooth muscle relaxation, is the 5-HT_7 receptor.

Use of a lower-case notation to describe a putative receptor defined only by gene product, with 'promotion' to a upper-case notation when the receptor is fully defined in terms of operational, recognitory and structural properties.

Up to 7 functional isoforms of the 5-HT_{2C} receptor, two functional isoforms of the 5-HT_4 receptor and four isoforms of the 5-HT_7 receptor were recognized.

Murphy et al. (1999) reviewed molecular biology-based alterations in 5-HT receptors including altered characteristics of mice lacking different 5-HT receptors, e.g., 5-HT_{1B}-receptor-deficient mice, 5-HT_{2C} receptor-deficient mice, 5-HT_{1A}-receptor-deficient mice, 5-HT cell-membrane-transporter deficient mice and vesicular monoamine-transporter-deficient mice.

The role of 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2B}, and 5-HT_7 receptors in cardiovascular physiology and pharmacology was discussed by Watts and Cohen (1999).

5-HT receptor types and subtypes

5-HT_1 subtypes. At least five *5-HT_1 subtypes* are described (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5HT_{1e}, 5HT_{1f}), which share 41–63% overall sequence identity and couple preferentially to $G_{i/o}$ to inhibit cAMP formation.

Hartig et al. (1996) suggested a revised nomenclature for 5-HT_{1B}, 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor subtypes.

5-HT_{1A} receptor agonists were described by Sills et al. (1984), Porsolt et al. (1992), Foreman et al. (1994), Wolff et al. (1997), Hamon (1997), 5-HT_{1A} receptor antagonists by Allen et al. (1997), 5-$HT_{1B/D}$ receptor antagonists by de Vries et al. (1997), 5-HT_{1D} receptor agonists by Macor et al. (1994), van Lommen et al. (1995), Valentin et al. (1996), 5-HT_{1D} receptor antagonists by Clitherow et al. (1994), de Vries et al. (1996), Rollema et al. (1996), Briley et al. (1997).

Presynaptic receptors may be preferably involved in the anxiolytic effects of 5-HT_{1A} receptor agonists, whereas in the antidepressant effects postsynaptic receptors are strongly involved (De Vry 1991, 1995).

5-HT_1 receptors are involved in learning and memory processes (see F.3.3.7 and F.3.4.1).

An endogenous peptide interacting specifically with the serotonergic 1B receptor subtypes was identified (Rousselle et al. 1996).

5-HT autoreceptors, mainly of the 5-HT_{1D} subtype, were studied by Starke et al. (1989), Fink et al. (1995), Bühlen et al. (1996), Glennon et al. (1996), Roberts et al. (1996), Price et al. (1996).

Cushing et al. (1994) studied the role of a 5-$HT_{1D\text{-}like}$ receptor in serotonin-induced contraction of canine coronary artery and saphenous vein.

See E.2.1.5.2 and E.2.1.5.3 for binding assays of 5-HT_1 receptors.

5-HT_2 receptors. *5-HT_2 receptors* have been subdivided into 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, which exhibit 46–50% overall sequence identity and couple preferentially to $G_{q/11}$ to increase the hydroly-

sis of inositol phosphates and elevate cytosolic [Ca^{2+}]. (Humphrey et al. 1993; Hoyer et al. 1994; Kebabian and Neumeyer 1994; Martin and Humphrey 1994; Saxena 1994; Shih et al. 1994; Tricklebank 1996; Martin 1998). The human 5-HT_2 receptors were cloned and characterized (Chen et al. 1992; Carey et al. 1996). Species differences in receptors were described (Johnson et al. 1995). 5-HT_2 receptors play a role in the action of antipsychotics and hallucinogens.

See E.5.1.9. and E.5.1.10 for binding assays to 5-HT_2 receptors.

5-HT_3 receptor. The *5-HT_3 receptor* is a pentameric, ligand-gated ion channel, activation of which promotes entry of Na^+ and Ca^{2+}, egress of K^+ and hence neuronal depolarization. It belongs to the transmitter-gated cation super-family of receptors and appears to be located exclusively in neuronal tissue where it mediates fast depolarization (Malone et al. 1991). Responses are blocked by a wide range of potent antagonists, which are highly selective with respect to other 5-HT receptors (Silverstone and Greenshaw 1996). The Bezold-Jarisch-reflex can be elicited by 5-HT_3 receptor agonists and be blocked by 5-HT_3 antagonists (see A.1.3.19). 5-HT_3 receptor antagonists are used as anti-migraine drugs. 5-HT_3 receptors are involved in feeding behavior (see L.3.1.1) Furthermore, gastric emptying (see J.4.4.2) and emesis (see J.5.0.1, J.5.0.2, J.5.0.3 and J.5.0.4) can be influenced by 5-HT_3 antagonists.

See E.2.1.5.4 for binding assays to the 5-HT_3 receptor.

5-HT_4 receptors. The *5-HT_4 receptors* couple preferentially to G_S protein and activate adenylate cyclase, thereby increasing intracellular cAMP levels (Martin 1998). This induces long-term modulation of ion-channel activity, which is fundamental in learning and memory (Eglen et al. 1995; Eglen and Hedge 1996). Excitatory responses were found in guinea pig ileum and colon, inhibitory responses in rat esophagus (Ford and Clarke 1993).

The tunica muscularis mucosae preparation of the rat esophagus has been recommended for evaluation of 5-HT_4 receptor ligands since it possesses a homogeneous population of 5-HT_4 receptors which mediates a well defined relaxant response to 5-HT (see J.2.0.1)

5-HT_4 receptors may mediate arrhythmias (Kaumann 1994). Two splice variants have been identified (Gerald et al. 1995). Several selective 5-HT_4 receptors agonists and antagonists were described (Gaster et al. 1995; Eglen and Hedge 1996; Eglen 1997).

Radioligand binding assays for 5-HT_4 receptors using [^{3}H]-GR113 808 have been described by Grossman et al. (1993), Domenech et al. (1994), Schiavi et al. (1994), Katayama et al. (1995), Ansanay et al. (1996).

A survey on molecular biology and potential functional role of **5-HT_5, 5-HT_6, and 5-HT_7 receptors** was given by Branchek and Zoombick (1997).

5-HT_6. The rat *5-HT_6* receptor has been cloned by Ruat et al. (1993). The distinguishing features of this receptor are the high affinity for a series of antipsychotic compounds as well as affinity for a number of cyclic antidepressants (Branchek 1995).

Bourson et al. (1995) used antisense oligonucleotides to determine the role of the 5-HT_6 receptor in the rat brain.

Boess et al. (1998) reported labelling of 5-hydroxytryptamine binding sites in rat and porcine striatum by the 5-HT_6 receptor-selective ligand [^{3}H]Ro 63-0563.

5-HT_7. Lovenberg et al. (1993), Shen et al. (1993), Gobbi et al. (1996), Villalón et al. (1997) described an adenyl cyclase-activating serotonin receptor (*5-HT_7*) implicated in the regulation of mammalian circadian rhythms.

A receptor autoradiographic and hybridization analysis of the distribution of the 5-HT_7 receptor in rat brain was reported by Gustafson et al. (1996).

Stowe and Barnes (1998) used [^{3}H]5-carboxamidotryptamine for selective recognition sites in rat brain.

Three different splice variants of the 5-HT_7 receptor have been described both in rat and human tissues (Vanhoenacker et al. 2000).

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E.2.1.5.2 Serotonin (5-HT_{1A}) receptor: binding of [^{3}H]-8-hydroxy-2-(di-n-propylamino)-tetralin ([^{3}H]-DPAT)

PURPOSE AND RATIONALE

Determination of the affinity of test compounds for the 5-HT_{1A} receptor in brain may be useful for predicting compounds with novel anxiolytic or atypical anti-psychotic profiles.

The existence of at least two populations of 5-HT_1 receptors in rat brain was shown by differential sensitivity to spiroperidol (Pedigo et al. 1981). The spiroperidol-sensitive receptors were designated as the 5-HT_{1A} subtype and the insensitive receptors were referred to as the 5-HT_{1B} subtype (Middlemis and Fozard 1983). Other 5-HT binding sites (5-HT_{1C}, 5-HT_{1D}, 5-HT_3 and 5-HT_4) have subsequently been identified in various species, based on differential sensitivity to 5-HT antagonists (Peroutka 1988).

Schlegel and Peroutka (1986) identified [^{3}H]DPAT as a selective ligand for the 5-HT_{1A} receptor. These authors reported that [^{3}H]DPAT labeled an autoreceptor. Lesion studies suggest that [^{3}H]DPAT labeled receptors are not terminal autoreceptors, but may be somatodendritic autoreceptors (Gozlan et al. 1983). Although DPAT decreases the firing rate in the raphe nucleus and inhibits 5-HT release, the actual location and function is somewhat controversial (Verge et al. 1986). These studies and the sensitivity of [^{3}H]DPAT binding to guanine nucleotides and effects on adenylate cyclase suggest that DPAT acts as an agonist at the 5-HT_{1A} receptor (Schlegel and Peroutka 1986).

Serotonin may play a role in anxiety, since drugs which reduce serotoninergic function have anxiolytic effects in animal models (Dourish et al. 1986). Since buspirone and its analogs have relatively higher affinity for the 5-HT_{1A} receptor than other receptors and no effect on the benzodiazepine site, their anxiolytic properties are attributed to activity at the 5-HT_{1A} receptor (Verge et al. 1986; Iversen 1984; Traber and Glaser 1987).

Besides 5-HT_{1A} receptor agonists (Misslin et al. 1990; Griebel et al. 1992; Hascoet et al. 1994; Stanhope and Dourish 1996), 5-HT_{1A} receptor antagonists (Traber and Glaser 1987; Cao and Rodgers 1998), 5-HT_{2A} receptor antagonists (Griebel 1996), 5-HT_{2C} receptor antagonists (Jenck et al. 1998), mixed receptor agonists/antagonists (Kleven et al. (1997), 5-HT_3 re-ceptor antagonists (Artais et al. 1995; Roca et al. 1995) and 5-HT_4 receptor antagonists (Kennett et al. 1997) exhibit anxiolytic properties (Handley and McBlane 1993).

Fletcher et al. (1995) described visualization and characterization of 5-HT receptors and transporters *in vivo* and in man.

PROCEDURE

Reagents

1. Tris buffers, pH 7.7
   a) 57.2 g Tris HCl
      16.2 g Tris base
      q.s. to 1 liter with distilled water
      (0.5 M Tris buffer, pH 7.7)
   b) Make a 1 : 10 dilution in deionized H_2O
      (0.05 M Tris buffer, pH 7.7)
   c) 0.05 M Tris buffer, pH 7.7 containing
      10 µM pargyline, 4 mM $CaCl_2$ and
      0.1% ascorbic acid.
      0.49 mg pargyline HCl
      111 mg $CaCl_2$
      250 mg ascorbic acid
      q.s. to 250 ml with 0.05 M Tris buffer,
      pH 7.7 (reagent 1b)
2. [^{3}H]-DPAT (2-(N,N-Di[2,3(n)-^{3}H]propylamino)-8-hydroxy-1,2,3,4-tetrahydronaphthalene) (160–206 Ci/mmol) was obtained from Amersham.
   For IC_{50} determinations: a 10 nM stock solution is made up and 50 µl are added to each tube (final concentration = 0.5 nM).
3. Serotonin creatinine sulfate. 0.5 mM stock solution is made up in 0.01 N HCl and 20 µl added to 3 tubes for determination of nonspecific binding (final concentration = 10 µM).
4. Test compounds:
   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 2×10^{-5} to 2×10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used based on the potency of the drug.

Tissue preparation

Male Wistar rats are sacrificed by decapitation. Hippocampi are removed, weighed and homogenized in 20 volumes of 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48 000 *g* for 10 min and the supernatant is discarded. The pellet is resuspended in an equal volume of 0.05 M Tris buffer, incubated at 37 °C for 10 min and recentrifuged at 48 000 *g* for 10 min. The final membrane pellet is resuspended in 0.05 M Tris buffer containing 4 mM $CaCl_2$, 0.1% ascorbic acid and 10 µM pargyline.

Assay

800 µl Tissue
130 µl 0.05 M Tris + $CaCl_2$ + pargyline + ascorbic acid
20 µl vehicle/5-HT/drug
50 µl [^{3}H]DPAT

Tubes are incubated for 15 min at 25 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 2 times with 5 ml of ice-cold 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml of Liquiscint scintillation cocktail and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 10 µM 5-HT. IC_{50} values are calculated from the percent specific binding at each drug concentration. The K_i value may then be calculated by the Cheng-Prusoff equation:

$$K_i = IC_{50} / 1 + L / K_D$$

The K_D value for [^{3}H] DPAT binding was found to be 1.3 nM by Scatchard analysis in a receptor saturation experiment.

MODIFICATIONS OF THE METHOD

Yocca et al. (1987) described BMY 7 378, a buspirone analog with high affinity, selectivity and low intrinsic activity at the 5-HT_{1A} receptor in rat and guinea pig hippocampal membranes.

Instead of the selective 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)-tetralin (DPAT), a selective antagonist to the 5-HT_{1A} receptor, 4-(methoxyphenyl)-1-[2'-(n-2"-pyridinyl)-p-iodobenzamido]-ethyl-piperazine ([^{125}I]p-MPPI) has been recommended (Kung et al. 1994a,b, 1995).

Several other selective 5-HT_{1A} receptor radioligands were recommended:

[^{3}H]lisuride (Sundaram et al. 1995); [^{3}H]WAY-100 635 ([O-methyl-^{3}H]-N-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-(2-piridinyl)cyclohexane carboxamide trihydrochloride) (Laporte et al. 1994; Gozlan et al. 1995; Hume et al. 1995; Khawaja 1995; Khawaja et al. 1997); [^{3}H]Alnespirone (Fabre et al. 1997); [Carbonyl^{11}C]-Desmethyl-WAY 100 635 (Pike et al. 1998); [^{3}H]S 15 535 (4-(benzodioxan-5-yl)-1-(indan-2-yl)piperazine) (Newman-Tancredi et al. 1998a); NAD-299 (=(R)-3-N,N-dicyclobutylamino-8-fluoro-[6-^{3}H]-3,4-dihydro-2H-1-benzopyran-carboxamide) (Jerning et al. 1998; Sandell et al. 1999).

Newman-Tancredi et al. (1998b) performed autoradiographic studies with [^{35}S]-GTPγS and the selective radioligand [^{3}H]S 15 535 for parallel evaluation of localization and functionality of the 5-HT_{1A} receptor.

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E.2.1.5.3 Serotonin (5-HT$_{1B}$) receptors in brain: binding of [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT)

PURPOSE AND RATIONALE

The purpose of this assay is to determine the affinity of test compounds for the serotonin (5-HT$_{1B}$) receptor in brain.

The existence of two populations of 5-HT$_1$ receptors in rat brain was shown by differential sensitivity to spiroperidol (Pedigo et al. 1981). The spiroperidol-sensitive receptors were designated as the 5-HT$_{1A}$ subtype and the insensitive receptors were referred to as the 5-HT$_{1B}$ subtype (Middlemiss and Fozard 1983). The 5-HT$_{1B}$ subtype has been identified in the brain of rats and mice (Peroutka 1986) and can be selectively labeled by 5-HT in rat striatum when spiroperidol is included to mask the 5-HT$_{1A}$ and 5-HT$_2$ receptors. In contrast to the situation in rats and mice, [^{3}H]5-HT binding in the basal ganglia of other mammals displays a pharmacological profile characteristic of 5-HT$_{1D}$ sites. The distribution of 5-HT$_{1B}$ sites in rat brain is similar to that of 5-HT$_{1D}$ sites in human brain (Segu et al. 1991; Boulenguez et al. 1992; Palacios et al. 1992).

By comparing the results in the 5-HT$_{1B}$ assay with those in the 5-HT$_{1A}$, 5-HT$_2$ and the 5-HT$_3$ receptor binding assays the relative affinity of a test compound for the major subclasses of 5-HT receptors in the rat brain can be determined.

PROCEDURE

Reagents

1. Tris buffers, pH 7.7
   a) 57.2 g Tris HCl
      16.2 g Tris base
      q.s. to 1 liter with distilled water
      (0.5 M Tris buffer, pH 7.7)
   b) Make a 1 : 10 dilution in deionized H_2O
      (0.05 M Tris buffer, pH 7.7)
   c) 0.05 M Tris buffer, pH 7.7 containing 10 mM pargyline, 4 mM $CaCl_2$ and 0.1% ascorbic acid.
      0.49 mg pargyline HCl
      110.99 mg $CaCl_2$
      250 mg ascorbic acid
      q.s. to 250 ml with 0.5 M Tris buffer, pH 7.7.
2. 5-Hydroxy[G-^{3}H]tryptamine creatinine sulfate (17–20 Ci/mmol) (Amersham). For IC_{50} determinations: a 40 nM stock solution is made up and 50 ml added to each tube (final concentration = 2.0 nM).
3. Serotonin creatinine sulfate. A 0.5 mM stock solution is made up in 0.01 N HCl and 20 ml added to 3 tubes for determination of nonspecific binding (final concentration = 10 µM).
4. Spiroperidol is dissolved in dilute glacial acetic acid. A 20 µM stock solution is prepared and 50 µl added to each tube to prevent binding to 5-HT$_{1A}$ and 5-HT$_2$ receptors (final concentration in the assay = 1 µM).
5. Test compounds:
   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2×10^{-5} to 2×10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used based on the potency of the drug.

Tissue preparation

Male Wistar rats are sacrificed by decapitation. Striata are removed, weighed and homogenized in 20 volumes of 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48 000 *g* for 10 min and the supernatant is discarded. The pellet is resuspended in an equal volume of 0.05 M Tris buffer, incubated at 37 °C for 10 min and recentrifuged at 48 000 *g* for 10 min. The final membrane pellet is resuspended in 0.05 M Tris buffer containing 4 mM $CaCl_2$, 0.1% ascorbic acid and 10 mM pargyline.

Assay

800 µl tissue
80 µl 0.05 M Tris + $CaCl_2$ + pargyline + ascorbic acid
20 µl vehicle/ 5-HT/ drug
50 µl [^{3}H]5-HT
50 µl spiroperidol

Tubes are incubated for 15 min at 25 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 2 times with 5 ml of ice-cold 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml of Liquiscint scintillation cocktail and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 10 µM 5-HT. IC_{50} values are calculated from the percent specific binding at each drug concentration. The K_i value may then be calculated by the Cheng-Prusoff equation:

$$K_i = IC_{50} / 1 + L / K_D$$

The K_D value for [^{3}H] 5-HT binding was found to be 16.5 nM by Scatchard analysis of a receptor saturation experiment.

MODIFICATIONS OF THE METHOD

[^{3}H]CP-96,501 = 3-(1,2,5,6-Tetrahydro-4-pyridyl-5-n-propoxyindole was recommended as a selective 5-HT_{1B} receptor radioligand (Koe et al. 1992; Lebel and Koe 1992).

Domenech et al. (1997) characterized human serotonin 1D and 1B receptors using [^{3}H]-GR-125 743, a novel radiolabelled serotonin 5-$HT_{1D/1B}$ receptor antagonist.

[^{3}H]mesulergine has been used as radioligand for 5-HT_{1C} receptor binding (Jenck et al. 1993, 1994).

[^{3}H]-5-carboxytryptamine was recommended as label for 5-HT_{1D} binding sites (Mahle et al. 1991; Novak et al. 1993).

Massot et al. (1998) described molecular, cellular and physiological characteristics of 5-HT-moduline, a tetrapeptide (Leu-Ser-Ala-Leu), acting as endogenous modulator of the 5-HT_{1B} receptor subtype.

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E.2.1.5.4 5-HT$_3$ receptor in rat entorhinal cortex membranes: binding of [^{3}H]GR 65630

PURPOSE AND RATIONALE

The purpose of this assay is to determine the affinity of test compounds for the 5-HT$_3$ binding site in the brain. This assay may be useful for predicting the potential of compounds to exhibit anti-emetic, anxiolytic or atypical antipsychotic profiles.

The 5-HT$_3$ binding site has also been characterized on operational, transductional and structural characteristics. Originally it was believed that 5-HT$_3$ binding sites existed only in the periphery (Costall et al. 1988). However, with the introduction of potent and selective 5-HT$_3$ antagonists such as GR65630, zacopride, ICS 205 930 and MDL 72222, and agonists, data from binding studies have indicated that 5-HT$_3$ binding sites are also located in selected areas of the brain (Kilpatrick et al. 1987, 1989; Barnes et al. 1988, 1990, 1992; Watling et al. 1988; Miller et al. 1992). The highest levels of 5-HT$_3$ binding sites have been detected in limbic and dopamine containing brain areas (entorhinal cortex, amygdala, nucleus accumbens and tuberculum olfactorium) (Costall et al. 1988). Besides possessing selective binding in dopamine rich areas, 5-HT$_3$ antagonists have been reported to block behavioral effects associated with certain drugs of abuse (nicotine and morphine) and to be active in behavioral tests predictive of anxiolytic activity. Based on these selective regional binding results and behav-ioral studies, it has been speculated that 5-HT$_3$ antagonists may have a therapeutic benefit in disease states believed to be associated with excessive dopaminergic activity; i.e., schizophrenia, anxiety and drug abuse.

Several authors described synthesis, pharmacology and therapeutic potential of H$_3$ receptor agonists and antagonists (Leurs et al. 1995, 1998; Stark et al. 1996).

PROCEDURE

Reagents

1. 0.05 M Krebs-HEPES buffer, pH 7.4
   11.92 g HEPES
   10.52 g NaCl
   0.373 g KCl
   0.277 g $CaCl_2$
   0.244 g $MgCl_2 \cdot 6H_2O$
   q.s. to 1 liter with distilled H_2O
   bring pH up to 7.4 (at 4 °C) with 5N NaOH
2. [^{3}H]GR65 630 (87.0 Ci/mmol) is obtained from New England Nuclear. For IC_{50} determinations: [^{3}H]GR65 630 is made up to a concentration of 1.0 nM in Krebs-HEPES buffer such that when 100 µl is added to each tube a final concentration of 0.4 nM is attained in the 250 µl assay.
3. Ondansetron HCl (GR 38 032F) is made up to a concentration of 500 µM in Krebs-HEPES buffer. 50 µl is added to each of 3 tubes for the determination of nonspecific binding (yielding a final concentration of 100 µM in the 250 µl assay.
4. Test compounds. For most assays, a 50 µM stock solution is made up in a suitable solvent and serially diluted with Krebs-HEPES buffer such that when 50 µl of drug is combined with the total 250 µl assay, a final concentration from 10^{-5} to 10^{-8} M is attained. Characteristically seven concentrations are studied for each assay; however, higher or lower concentrations may be used, depending on the potency of the drug.

Tissue preparation

Male Wistar rats (150–200 g) are decapitated, the entorhinal cortex removed, weighed and homogenized in 10 volumes of ice-cold 0.05 M Krebs-HEPES buffer, pH 7.4. The homogenate is centrifuged at 48 000 *g* for 15 min at 4 °C. The resulting pellet is rehomogenized in fresh Krebs-HEPES buffer and recentrifuged at 48 000 *g* for 15 min at 4 °C. The final pellet is resuspended in the original volume of ice-cold Krebs-HEPES buffer. This yields a final tissue concentration of 1.2–1.6 mg/ml with the addition of 100 ml to the assay. Specific binding is approximately 55–65% of total bound ligand.

Assay

100 µl Tissue suspension
100 µl [^{3}H]GR65 630
50 µl Vehicle (for total binding) or 500 mM ondansetron HCl (for nonspecific binding) or appropriate drug concentration

Sample tubes are kept on ice for additions, then vortexed and incubated with continuous shaking for 30 min at 37 °C. At the end of the incubation period, the incubate is diluted with 5 ml of ice-cold Krebs-HEPES buffer and immediately vacuum filtered through What-man GF/B filters, followed by two 5-ml washes with ice-cold Krebs-HEPES buffer. The filters are dried and counted in 10 ml of liquid scintillation cocktail.

EVALUATION

Specific GR65 630 binding is defined as the difference between the total binding and that bound in the presence of 100 µM Ondansetron HCl. IC_{50} calculations are performed using computer-derived log-probit analysis.

MODIFICATIONS OF THE METHOD

Dunn et al. (1991) found that preclinical data with 5-HT$_3$ antagonists predict anxiolytic rather than antipsychotic activity.

Davies et al. (1999) found that the 5-HT$_{3B}$ subunit is a major determinant of serotonin receptor function.

Reiser and Hamprecht (1989) reported that substance P and serotonin (via 5-HT$_3$ receptors) act synergistically to activate the cation permeability as measured by [^{14}C]guanidinium uptake in neuroblastoma × glioma hybrid cells.

Bönisch et al. (1993) studied the 5-HT$_3$ receptor-mediated cation influx into N1E-115 mouse neuroblastoma cells by the use of the organic cation [^{14}C]-guanidinium.

Emerit et al. (1993) assessed the [^{14}C]guanidium accumulation in cells of the hybridoma (mouse neuroblastoma × rat glioma) clone NG 108-15 exposed to serotonin 5-HT$_3$ receptor ligands and substance P.

Bonhaus et al. (1993) characterized the binding of [^{3}H]endo-N-(8-methyl-8-azabicyclo[3.2.1.]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazolone-1-carboxamide hydrochloride ([^{3}H]BIMU-1), a benzimidazolone with high affinity to 5-HT$_3$ and 5-HT$_4$ receptors in NG 108 cells and guinea pig hippocampus.

Kooyman et al. (1994) studied the specific binding of [^{3}H]GR65 630 to 5-HT$_3$ recognition sites in cultured N1E-115 mouse neuroblastoma cells.

Several other selective 5-HT$_3$ receptor radioligands were recommended:

[^{3}H]Quipazine (Perry 1990); tritium-labeled 1-methyl-N-[8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1H-indazole-3-carboxamide (Robertson et al. 1990); [^{125}I]-(S)-iodozacopride (Gehlert et al. 1993); [^{3}H]-BRL46 470 (Steward et al. 1995); [^{3}H]YM060 (Azukawa et al. 1995); (^{125}I)iodophenpropit (Jansen et al. 1994), (^{125}I)iodoproxyfan (Ligenau et 1994), (S)-Des-4-amino-3-[^{125}I]iodozacopride (Mason et al. 1996; Hewlett et al. 1999).

Tairi et al. (1998) and Hovius et al. (1999) studied ligand binding to the serotonin 5-HT$_3$ receptor with a novel fluorescence ligand and developed a total internal reflection fluorescence assay which is suitable for high-through-put screening.

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E.2.1.6 Histamine H$_3$ receptor binding in brain

PURPOSE AND RATIONALE

Histamine modulates its own synthesis and release from depolarized brain slices or synaptosomes by interacting with H$_3$ autoreceptors with a pharmacology distinct from that of H$_1$ and H$_2$ receptors (Arrang et al. 1985, 1987, 1990; Hill 1990, 1992; Hill et al. 1997; Leurs et al. 1991, 1998). The R-isomer of α-methylhistamineα (α-MeHA) was identified as a highly selective H$_3$-receptor agonist active at nanomolar concentrations. Furthermore, this compound in ^{3}H-labeled form is a suitable probe for the H$_3$-receptor.

PROCEDURE

The cerebral cortex from guinea pigs is dissected and homogenized in 50 volumes ice-cold 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, in a Potter homogenizer. The

homogenate is centrifuged for 15 min at 750 g. The pellet is discarded and the supernatant centrifuged at 42 000 *g* for 15 min. The supernatant is discarded and the pellet washed superficially with and then resuspended in fresh buffer. The protein concentration of the membrane suspension as determined according to Lowry et al. (1951) is about 0.3–0.4 mg/ml.

Aliquots of the membrane preparation are incubated for 60 min at 25 °C with ^{3}H(R) α-MeHA and unlabeled substances in a final volume of 1 ml. The assay is stopped by dilution with 2 × 3 ml ice-cold medium, followed by rapid filtration under vacuum over Millipore AAWP filters which are then rinsed twice with 5 ml of ice-cold medium. Radioactivity retained on the filters is measured by liquid scintillation spectroscopy.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 10 μM unlabeled α-MeHA. IC_{50} values are calculated from the percent specific binding at each drug concentration. The K_i value may then be calculated by the Cheng-Prusoff equation:

$$K_i = IC_{50} / 1 + L / K_D$$

MODIFICATIONS OF THE METHOD

Jansen et al. (1992) described [^{125}I]iodophenpropit as a radiolabeled histamine H_3 receptor antagonist.

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E.2.2 Anticonvulsant activity

E.2.2.1 Pentylenetetrazole (Metrazol) induced convulsions

PURPOSE AND RATIONALE

This assay has been used primarily to evaluate antiepileptic drugs. However, it has been shown that most anxiolytic agents are also able to prevent or antagonize Metrazol-induced convulsions.

PROCEDURE

Mice of either sex with a body weight between 18 and 22 g are used. The test compound or the reference drug is injected sc. or i.p. or given orally to groups of 10 mice. Another group of 10 mice serves as control. Fifteen min after sc.-injection, 30 min after i.p.-injection, or 60 min after oral administration 60 mg/kg MTZ (Metrazol) are injected subcutaneously. Each animal is placed into an individual plastic cage for observation lasting 1 h. Seizures and tonic-clonic convulsions are recorded. At least 80% of the animals in the control group have to show convulsions.

EVALUATION

The number of protected animals in the treated groups is calculated as percentage of affected animals in the

control group. ED_{50}-values can be calculated. Furthermore, the time interval between MTZ-injection and occurrence of seizures can be measured. The delay of onset is calculated in comparison with the control group.

CRITICAL ASSESSMENT OF THE METHOD

The method is widely accepted as a screening procedure and has been modified by many investigators. Chlordiazepoxide (20 mg/kg i.p.), diphenylhydantoin (10 mg/kg i.p.) and phenobarbitone sodium (20 mg/kg i.p.) were found to be effective. Predominantly, the muscle relaxant and anticonvulsant effects of benzodiazepines are measured by this test. Stimulant, anti-depressant, neuroleptic and some anti-epileptic drugs do not show MTZ-antagonism at tolerable doses (Lippa et al. 1979). Nevertheless, the antagonism of MTZ-induced seizures appears to be a suitable procedure for detecting compounds with potential anxiolytic activity. Among a battery of tests, the MTZ-antagonism has been proposed to study centrally acting skeletal muscle relaxants (Bastian et al. 1959; Domino 1964).

MODIFICATIONS OF THE METHOD

Different routes of administration (i.p., i.v., s.c.) have been used by various investigators. Moreover, the dose of MTZ which causes seizures in 80 to 90% of the animals varies with the strain being used.

Bastian et al. (1959) published a modification, whereby mice are infused with a MTZ-solution through a small-diameter polyethylene tubing into the tail vein. In the same animal 3 end-points are registered: 1. Onset of persistent clonic convulsions, 2. beginning of the tonic flexor phase, and 3. time to death. The three endpoints are affected differently by various drugs providing the basis for the determination of drug specificity.

Löscher et al. (1991) tested 8 clinically established antiepileptic drugs in three pentylentetrazole seizure models: (1) the threshold for different types of pentylentetrazole seizures, i.e., initial myoclonus twitch, generalized clonus with loss of righting reflexes and tonic backward extension of forelimbs (forelimb tonus) in mice; (2) the traditional pentylentetrazole seizure test with s.c. injection of the CD97 for generalized clonic seizures in mice; and (3) the s.c. pentylentetrazole seizure test in rats. Various factors may cause misleading predictions from pentylentetrazole seizure models.

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E.2.2.2 Strychnine-induced convulsions

PURPOSE AND RATIONALE

The convulsing action of strychnine is due to interference with postsynaptic inhibition mediated by glycine. Glycine is an important inhibitory transmitter to motoneurons and interneurons in the spinal cord, and strychnine acts as a selective, competitive antagonist to block the inhibitory effects of glycine at all glycine receptors. Strychnine-sensitive postsynaptic inhibition in higher centers of the CNS is also mediated by glycine. Compounds which reverse the action of strychnine have been shown to have anxiolytic properties.

PROCEDURE

Groups of 10 mice of either sex with a weight between 18 and 22 g are used. They are treated orally with the test compound or the standard (e.g. diazepam 5 mg/kg). One hour later the mice are injected with 2 mg/kg strychnine nitrate i.p. The time until occurrence of tonic extensor convulsions and death is noted during a 1 h period. With this dose of strychnine convulsions are observed in 80% of the controls.

EVALUATION

ED_{50}-values are calculated using various doses taking the percentage of the controls as 100%. For time-response curves the interval between treatment and strychnine injection varies from 30 to 120 min.

CRITICAL ASSESSMENT OF THE METHOD

The method has been proven to be useful in a battery of tests to characterize CNS-active drugs. (Costa et al. 1975).

MODIFICATIONS OF THE METHOD

McAllister (1992) induced spinal seizures in mice by rotating them along the body axis clockwise and anticlockwise alternatively three times following pretreatment with a subconvulsive dose of strychnine.

Lambert et al. (1994) tested the antagonism of a glycine derivative against seizures induced by 3-mercaptopropionic acid (3-MPA).

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E.2.2.3 Picrotoxin-induced convulsions

PURPOSE AND RATIONALE
Picrotoxin induced convulsions are used to further evaluate CNS-active compounds. Picrotoxin is regarded as a $GABA_A$-antagonist modifying the function of the chloride ion channel of the $GABA_A$ receptor complex.

PROCEDURE
Groups of 10 mice of either sex with a weight between 18 and 22 g are treated either orally or i.p. with the test compound or the standard (e.g. 10 mg/kg diazepam i.p.). Thirty min after i.p. treatment or 60 min after oral administration the animals are injected with 3.5 mg/kg s.c. picrotoxin and are observed for the following symptoms during the next 30 min: clonic seizures, tonic seizures, death. Times of onset of seizures and time to death are recorded.

EVALUATION
For time-response curves the animals receive the drug 30, 60 or 120 min prior to picrotoxin. Protection is expressed as percent inhibition relative to vehicle control. The time period with the greatest percent inhibition is said to be the peak time of drug activity. ED_{50}-values are calculated taking the percentage of seizures in the control group as 100%.

CRITICAL ASSESSMENT OF THE METHOD
The method has been proven to be of value amongst a battery of tests for CNS-activity.

MODIFICATIONS OF THE METHOD
Buckett (1981) describes an intravenous bicuculline test in mice (see Sect. E.3.2.6). The compound bicuculline antagonizes the action of GABA by competition on postsynaptic receptors. In the whole animal bicuculline reproducibly induces myoclonic seizures. An intravenous dose of 0.55 mg/kg was found to induce myoclonic seizures in 90–100% of mice with less than 10% mortality. GABAergic compounds such as benzodiazepines at relatively low doses antagonize the bicuculline-induced myoclonic seizures.

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E.2.2.4 Isoniazid-induced convulsions

PURPOSE AND RATIONALE
Isoniazid can precipitate convulsions in patients with seizure disorders. The compound is regarded as a GABA-synthesis inhibitor (Costa et al. 1975). Clonic-tonic seizures are elicited in mice which are antagonized by anxiolytic drugs.

PROCEDURE
Ten mice of either sex with a weight of 18 to 22 g are treated with the test compound or the standard (e.g. diazepam 10 mg/kg i.p.) by oral or intraperitoneal administration. Controls receive the vehicle only. 30 min after i.p. or 60 min after p.o. treatment the animals are injected with a subcutaneous dose of 300 mg/kg isoniazid (isonicotinic acid hydrazide). During the next 120 min the occurrence of clonic seizures, tonic seizures and death is recorded.

EVALUATION
The percentage of seizures or death occurring in the control group is taken as 100%. The suppression of these effects in the treated groups is calculated as percentage of controls. ED_{50}-values are calculated.

CRITICAL ASSESSMENT OF THE METHOD
The method has been proven to be of value amongst a battery of tests for CNS-activity.

REFERENCES
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E.2.2.5 Yohimbine-induced convulsions

PURPOSE AND RATIONALE

Antagonism against yohimbine-induced seizures in mice is considered to be a model predictive of potential anxiolytic and GABA-mimetic agents (Dunn and Fielding 1987).

PROCEDURE

Male Swiss-Webster mice (20–30 g) are individually placed in clear plastic cylinders and test compounds are administered i.p. 30 min prior to 45 mg/kg s.c. of yohimbine HCl. The animals are observed for the onset and number of clonic seizures for 60 min.

EVALUATION

ED_{50} values with 95% confidence limits are calculated for the antagonism of yohimbine-induced clonic seizures by means of the Lichtfield-Wilcoxon procedure.

CRITICAL ASSESSMENT OF THE METHOD

The antagonism against yohimbine-induced seizures can be regarded as an useful test amongst a battery of tests for anxiolytic activity.

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Dunn R, Fielding S (1987) Yohimbine-induced seizures in mice: A model predictive of potential anxiolytic and GABA-mimetic agents. Drug Dev Res 10:177–188

Dunn RW, Corbett R (1992) Yohimbine-induced convulsions involve NMDA and GABAergic transmission. Neuropharmacol 31:389–395

Dunn RW, Corbett R, Martin LL, Payack JF, Laws-Ricker L, Wilmot CA, Rush DK, Cornfeldt ML, Fielding S (1990) Preclinical anxiolytic profiles of 7189 and 8319, novel non-competitive NMDA antagonists. Current and Future Trends in Anticonvulsant, Anxiety, and Stroke Therapy, Wiley-Liss, Inc., pp 495–512

E.2.3 Anti-aggressive activity

E.2.3.1 Foot-shock induced aggression

PURPOSE AND RATIONALE

Since the discovery of the taming effects of benzodiazepines in vicious monkeys (Heise and Boff 1961; Randall et al. 1961) tests for agents with anti-aggressivity activity have been developed for various animal species. These tests include foot-shock induced aggression in mice and rats, fighting behavior of isolated mice and aggressiveness of rats which become extremely vicious after lesions in the septal area of the brain (Brady and Nauta 1953).

Foot-shock induced aggression is used for further characterization of centrally active drugs. Irwin et al. (1971) have attempted to compare drug classes with this method.

PROCEDURE

Male mice (NMRI, Ivanovas) with a weight between 20 and 30 g are used. Two mice are placed in a box with a grid floor consisting of steel rods with a distance of 6 mm. A constant current of 0.6 or 0.8 mA is supplied to the grid floor by a LVE constant current shocker with an associated scrambler. A 60-Hz current is delivered for 5 s followed by 5 s. intermission for 3 min. Each pair of mice is dosed and tested without previous exposure. The total number of fights are recorded for each pair during the 3-min period. The fighting behavior consists of vocalization, leaping, running, rearing and facing each other with some attempt to attack by hitting, biting or boxing. The test compound or the standard are applied either 30 min before the test i.p. or 60 min before the test orally. For a time response, the drug is given 30, 60 and 120 min prior to testing. Six pairs of drug-treated and two pairs of vehicle-treated animals are utilized for each time period. A dose range is tested at the peak of drug activity. A minimum of 3 doses (10 pairs of mice/dose) is administered for a range of doses. Control animals receive the vehicle.

EVALUATION

The percent inhibition of aggression is calculated from the vehicle control. ED_{50}-values are calculated.

CRITICAL ASSESSMENT OF THE METHOD

Not only anxiolytics but also other classes of drugs where found to be active in this test such as sedatives like meprobamate and phenobarbital, neuroleptics such as perphenazine, analgesics such as methadone, and ethyl alcohol.

MODIFICATIONS OF THE METHOD

A survey of aggressive behavior in the **rat** has been given by Blanchard and Blanchard (1977).

Induction of aggressive behavior by electrical stimulation in the hypothalamus of male rats was described by Kruk et al. (1979).

Mos and Olivier (1991) reviewed the concepts in animal models for pathological aggressive behavior in human.

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E.2.3.2 Isolation-induced aggression

PURPOSE AND RATIONALE

Male mice, submitted to prolonged isolation, develop aggressive behavior against animals of the same sex. Compounds can be tested for their ability to suppress this isolation-induced aggression.

PROCEDURE

Male mice of NMRI-strain with an initial weight of 12 g are kept isolated in small Makrolon®-cages for a period of 6 weeks. Prior to the administration of the test drug, the aggressive behavior of the animals is tested. A male mouse being accustomed to live together with other animals is placed into the cage of an isolated mouse for 5 min. Immediately, the isolated mouse will start to attack the "intruder". The aggressive behavior of the isolated mouse is characterized by hitting the tail on the bottom of the cage, screaming and biting. The reaction time until the first attack is less than 10 s for most of the isolated mice and relatively constant for one individual. After these initial tests, the isolated mice receive the test compound, the standard drug or the vehicle either orally or subcutaneously. The aggressive behavior is evaluated 60, 120 and 240 min after oral and 30, 60 and 120 min after sc. treatment. After treatment with certain centrally acting compounds the aggressive behavior of the isolated mice is changed. The reaction time until the first attack can be prolonged or shortened. The fighting reaction can be attenuated. Then, additional mechanical stimuli can be used to elicit the fighting behavior. With highly effective drugs the aggressive behavior is completely suppressed.

EVALUATION

The number of animals with complete suppression of the fighting behavior is calculated. In animals with diminished aggressiveness the reaction time is registered. A gradual scale of inhibition of aggressiveness is established.

CRITICAL ASSESSMENT OF THE METHOD

The fighting behavior of isolated mice is not only altered by sedative and anxiolytic compounds but also by neuroleptics and antidepressants. For example, active doses are:

Lorazepam	2.5	mg/kg p.o.
Clonazepam	2.5	mg/kg sc.
Haloperidol	1.0	mg/kg p.o.
Chlorpromazine	10.0	mg/kg s.c.
Imipramine	25.0	mg/kg s.c.

In this way, anti-aggressive activity of several classes can be detected.

MODIFICATIONS OF THE METHOD

Krsiak (1974, 1979) described the effects of various drugs on behavior of aggressive **mice**.

Olivier and van Dalen (1982) discussed the social behavior in rats and mice as an ethologically based model for differentiating psychoactive drugs.

McMillen et al. (1987) tested the effects of drugs on aggressive behavior and brain monoaminergic neurotransmission.

Krsiak (1975) showed that about 45% of single-housed male mice showed timidity instead of aggres-

sion on interactions with group-housed male mice. Several drugs, such as benzodiazepines, chlorpromazine and barbitone inhibited the isolation induced timidity without reducing other motor activities in the timid mice.

Andrade et al. (1988) tested the effect of insulin-induced hypoglycemia on the aggressive behavior of isolated mice against intruders made anosmic by application of 25 µl of 4% zinc sulfate solution to the nasal tract 3 and 1 days before the encounters.

White et al. (1991) tested the effects of serotoninergic agents and other psychotropic compounds on isolation-induced aggression in mice at doses below those which produced debilitation in the rotarod performance.

Francès (1988), Francès et al. (1990), Francès and Monier (1991) described an other phenomenon of isolation in rodents which can be used for evaluation of psychotropic drugs: the **isolation-induced behavioral deficit**. Male Swiss NMRI mice were either housed in groups of 6 in home cages or isolated at the age of 4–5 weeks for 7 days. Mice were tested in pairs (one isolated and one grouped mouse) under a transparent beaker. The number of escapes was counted for the first 2 min of observation. An attempt to escape was defined as one of the following: (1) the two forepaws were placed against the wall of the beaker, (2) the mouse was sniffing, its nose at the spout of the beaker, (3) the mouse was scratching at the glass floor. Behavioral observations were taped by an observer, blind to the treatment.

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E.2.3.3 Resident-intruder aggression test

PURPOSE AND RATIONALE

Sijbesma et al. (1991), Mos et al. (1992), Muehlenkamp et al. (1995) studied the effects of drugs in a test for offensive aggression, the isolation-induced resident intruder aggression model (Flannelly and Lore 1975; Brain et al. 1979), in the rat.

PROCEDURE

Sprague-Dawley rats weighing 250 to 450 g are housed in a light-dark (12L:12D)-, temperature (ca. 22 °C)- and humidity (ca. 55%)-controlled room.

Resident male rats (about 450 g) are tested in their home cages for aggression against a smaller (250 g) male intruder. They are treated by intraperitoneal injection of test drug or saline 15 min before the test. The resident female is removed from the cage 30 min prior to the start of the test period. After placing the intruder rat in the territorial cage, the behavior of the resident male is observed. The time until the first attack (in seconds), number of attacks, and duration of each attack (in seconds) are recorded for the next 15 min by a blind observer. Furthermore, a total of 49 different behavioral elements are scored and grouped into 7 behavioral categories: offensive, exploration, social interest, inactivity, avoidance, body care, defense.

EVALUATION

Paired and unpaired *t*-tests are used for comparisons of means of absolute values.

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Brain PF, Howell PA, Benton D, Jones SE (1979) Studies on responses by "residents" rats housed in different ways to intruders of differing endocrine status. J Endocrinol 81: 135–136

Flannelly K, Lore R (1975) Dominace-subordinance in cohabitating pairs of adult rats: Effects on aggressive behavior. Aggress Behav 1:331–340

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Muehlenkamp F, Lucion A, Vogel WH (1995) Effects of selective serotonergic agonists on aggressive behavior in rats. Pharmacol Biochem Behav 50:671–674

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E.2.3.4 Water competition test

PURPOSE AND RATIONALE

The competitive water consumption test in rats (Baenninger 1970; Syme 1974) can be used to study the influence of drugs on defensive aggression and dominance (Muehlenkamp et al. 1995).

PROCEDURE

Male Sprague-Dawley rats weighing 250 to 400 g are kept in a light-dark (12L:12D)-, temperature (ca. 22 °C)- and humidity (ca. 55%)-controlled room.

Animals of equal weight are paired and housed in one cage. After 6 days, the animals are deprived of water for 23 h. Then, one water bottle is introduced with a shielded spout so that only one animal of a pair can drink at a time. Time (in seconds) and frequency of spout possession and water consumption are recorded in numbers with a special computer program for 5 min. Animals are then allowed another 55 min for water consumption. This test is repeated on 3 subsequent days. Saline or test substances are injected intraperitoneally 15 min before tests 3 and 4. The animal with the longest duration of water consumption and frequency of spout possession is considered to be the more aggressive and/or dominant animal.

EVALUATION

Paired and unpaired *t*-tests are used for comparisons of means of absolute values, as well as means of differences of the absolute value of the two rats.

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Muehlenkamp F, Lucion A, Vogel WH (1995) Effects of selective serotonergic agonists on aggressive behavior in rats. Pharmacol Biochem Behav 50:671–674

Syme GJ (1974) Competitive orders a measures of social dominance. Anim Behav 22:931–940

E.2.3.5 Maternal aggression in rats

PURPOSE AND RATIONALE

The model of 'maternal aggression' in rats was described by Olivier et al. (1985), Olivier and Mos (1986, 1992), Mos et al. (1987, 1989). Introduction of an intruder (male or female) in the cage of a parturient female rat, induces high levels of aggression against such intruders. Maternal aggression is characterized by short latency attacks of high intensity, mostly directed toward the head or neck of the intruder and is particularly pronounced during the first part of the lactating period. The behavior can be suppressed by several drugs, e.g., 5-HT_1 agonists (Mos et al. 1990; Olivier and Mos 1992).

PROCEDURE

Female rats weighing 250–350 g are placed with a breeding male in their home cages. On the bottom of each cage an iron gauze is placed which enables the collection of ejaculation plugs. After an ejaculation plug is detected the male is left for an other week with the female after which she is placed in the observation

cage provided with nesting material where she stays for the rest of the experiment. These cages are situated in an observation room under a reversed day-night rhythm. The day of birth is marked as day 0. Every parturient female is tested each day against a naive male intruder which has about 25 g less body weight than the female. Tests are performed during the first part of the dark period under red light conditions. One male intruder is placed in the female's home cage for 5 min. The ongoing behavior is videotaped and analyzed later. Each intruder is used only once and sacrificed immediately afterwards with an i.p. overdose of pentobarbital, followed by shaving and describing the wounds on wound charts (Mos et al. 1984).

The aggressive behavior of the female is scored for:

- bite attack on the head (fierce biting on head and snout often causing severe wounds),
- bite attack on the body (mostly directed to the back),
- lateral threat (the animal kicks with a hindleg at the opponent),
- upright posture (accompanied by boxing),
- nipping (short and low intensity bite on the head of the opponent),
- pulling (the opponent is held by the teeth and drawn through the cage),
- lunge (very rapid movement toward the opponent, mostly followed by bite attacks),
- on top (the female holds down the opponent which lies on its back).

Besides the frequency and the duration of the elements, also the latency of the first attack and the number of attacks are recorded.

Drug experiments are performed on postpartum days 3, 5, 7, and 9 using a Latin-square design of dosage. Preceding days (day 1 and 2), intervening (4, 6 and 8) and following (10, 11, 12, and 13) days are used to establish an aggression base line and as wash-out days. Drugs are administered orally 60 min before testing.

EVALUATION

Analysis of variance is employed to detect overall significance, followed by Wilcoxon matched pairs comparison between dosages. Kruskal-Wallis analysis is used to test the differences in the bite target areas after drug treatment.

MODIFICATIONS OF THE METHOD

Postpartum aggression in rats did not influence threshold currents for electrical brain stimulation-induced aggression in rats (Mos et al. 1987).

Maternal aggression can be observed not only in rats but also in **mice** and **hamsters** (Mos et al. 1990).

Palanza et al. (1996) examined the effects of chlordiazepoxide on the differential response pattern in aggressive-naive and aggressive-experienced lactating female mice confronting intruders of either sex in a 10-min test.

Olivier et al. (1990) highlighted ethopharmacology as a creative approach to identification and characterization of novel psychotropics.

Olivier et al. (1995) gave a review on serotonin receptors and animal models of aggressive behavior.

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E.2.3.6 Rage reaction in cats

PURPOSE AND RATIONALE

An emotional aggressive behavior (rage reaction) can be elicited in unrestrained cats by high frequency electrical stimulation of the hypothalamus. Benzodiazepines have been reported (Malick 1970; Mursaki et al. 1976) to elevate the stimulus threshold for eliciting this rage reaction.

PROCEDURE

Emotional aggressive behavior is evoked by stimulation of the perifornical area of the lateral hypothalamus through chronically implanted stainless steel bipolar concentric electrodes by using threshold impulses of 1.0–2.6 mA, delivered at 50 Hz, to evoke a control attack response. Stimulation is discontinued immediately after the slowly rising current has reached threshold strength, and is performed once before drug administration as well as every 30 min thereafter. Drugs are injected by intraperitoneal route.

EVALUATION

Post-drug values are expressed as percentage of the pre-drug control value. Student's paired *t*-test is used for each time interval.

CRITICAL ASSESSMENT OF THE TEST

The method has been used for evaluation of new drugs. Most studies were devoted to the role of neurotransmitters regulating feline aggression (Siegel and Schubert 1995; Siegel et al. 1998) and the neural bases of aggression and rage in the cat (Siegel et al. 1997, 1999).

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E.2.4 Effects on behavior

E.2.4.1 Anti-anxiety test (Light-dark model)

PURPOSE AND RATIONALE

Crawley and Goodwin (1980) Crawley (1981) described a simple behavior model in mice to detect compounds with anxiolytic effects. Mice and rats tend to explore a novel environment, but to retreat from the aversive properties of a brightly-lit open field. In a two-chambered system, where the animals can freely move between a brightly-lit open field and a dark corner, they show more crossings between the two chambers and more locomotor activity after treatment with anxiolytics. The numbers of crossings between the light and dark sites are recorded.

PROCEDURE

The testing apparatus consists of a light and a dark chamber divided by a photocell-equipped zone. A polypropylene animal cage, $44 \times 21 \times 21$ cm, is darkened with black spray over one-third of its surface. A partition containing a 13 cm long $\times$ 5 cm high opening separates the dark one third from the bright two thirds of the cage. The cage rests on an Animex® activity monitor which counts total locomotor activity. An electronic system using four sets of photocells across the partition automatically counts movements through the partition and clocks the time spent in the light and dark compartments. Naive male mice or rats are placed into the cage. The animals are treated 30 min before the experiment with the test drugs or the vehicle intraperitoneally and are then observed for 10 min. Groups of 6–8 animals are used for each dose.

EVALUATION

Dose-response curves are obtained and the number of crossings through the partition between the light and the dark chamber are compared with total activity counts during the 10 min.

CRITICAL ASSESSMENT OF THE METHOD

It has been shown that a variety of anxiolytics, including diazepam, pentobarbital and meprobamate produce

a dose-dependent increase in crossings, whereas non-anxiolytic agents do not have this facilitatory effect. Furthermore, the relative potency of anxiolytics in increasing exploratory behavior in the two-compartment chamber agrees well with the potency found in clinical trials.

The test has the advantage of being relatively simple with no painful stimuli to the animals. The specificity of the method remains open.

MODIFICATIONS OF THE METTHOD

Using a similar method, called black and white test box, Costall et al. (1987, 1988, 1989) studied the effects of anxiolytic agents and reported an anxiolytic effect of dopamine receptor antagonists, such as sulpiride and buspirone.

Sanchez (1995) presented a fully automated version of the black and white two-compartment box for mice.

Barnes et al. (1992) used this model to study the interaction of optical isomers modifying rodent aversive behavior.

Kilfoil et al. (1989) used a similar apparatus to test compounds for anxiogenic and anxiolytic activity.

Animal models of anxiety and their relation to serotonin-interacting drugs have been reviewed by Broekkamp et al. (1989) and by Griebel (1995).

Laboratory rats prefer to dwell on a solid floor rather than an grid one, particularly when resting. Manser et al. (1996) described an operant test in rats to determine the strength of preference for flooring. The apparatus consisted of a grid-floored cage and a solid-floored cage, joined via a central box containing a barrier whose weight was adjustable. The rats had to lift the barrier in order to explore the whole apparatus or were confined on the grid floor and then had to lift the barrier in order to reach the solid floor.

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E.2.4.2 Anticipatory anxiety in mice

PURPOSE AND RATIONALE

When group-housed mice are removed one by one from their home cage, the last mice removed have always higher rectal temperatures than those removed first (Borsini et al. 1989; Lecci et al. 1990). This phenomenon is interpreted as being caused by anticipatory fear for an aversive event (handling causes stress-induced hyperthermia). Consequently, this test is thought to be a model of anticipatory anxiety. The anticipatory increase in temperature was prevented by prior treatment with diazepam and buspirone, whereas several other drugs did not affect this phenomenon (Lecci et al. 1990). The usefulness of this model for obsessive-compulsive disorder in man is discussed.

PROCEDURE

Groups of 18 male albino Swiss mice weighing 25–30 g are housed at constant room temperature and relative

humidity for at least 7 days in Makrolon cages to adapt to the environment. Test drugs or standard (diazepam) or solvent are administered orally in various doses to groups of 18 mice prior to the test. Thirty min later, the first 3 mice are removed from the cage and the rectal temperature registered by inserting a silicone lubricated thermistor probe (2 mm diameter) for 2.5 cm into the rectum. The average temperature of these 3 mice is taken as basal value. Mice number 4 through 15 are simply removed and again returned to the cage, and thereafter body temperature is determined in the remaining three animals. The difference of the mean value of these mice and the basal values is calculated as increase. Vehicle treated test groups display increases of 1.1 to 1.3 °C.

EVALUATION

The mean increase values of treated groups ±SEM are compared by ANOVA statistics with the controls.

MODIFICATIONS OF THE METHOD

Van der Heyden et al. (1997) adapted the group-housed stress-induced hyperthermia paradigm to single housed animals in order to drastically reduce the number of animals used. Repeated, but not single, disturbance of animals resulted in a strong hyperthermia (Δt) within 10 min. The final test paradigm chosen involved repeated temperature measurements at 10 min intervals, thus providing both information on basal temperature and Δt in each animal within a short time frame.

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E.2.4.3 Social interaction in rats

PURPOSE AND RATIONALE

In an unfamiliar and brightly lit environment, the normal social interaction of rats (e.g. sniffing, nipping, grooming) is suppressed. Anxiolytics counteract this suppression.

PROCEDURE

Male Sprague-Dawley rats (225–275 g body weight) are housed in groups of 5 animals The apparatus used for the detection of changes in social behavior and exploratory behavior consists of a Perspex open-topped box (51×51 cm and 20 cm high) with 17×17 cm marked areas on the floor. One hour prior to the test, two naive rats from separate housing cages are treated with the test compound orally. They are placed into the box (with 60 W bright illumination 17 cm above) and their behavior is observed over a 10-min period by remote video recording. Two types of behavior can be noted:

- social interaction between the animals is determined by timing the sniffing of partner, crawling under or climbing over the partner, genital investigation of partner, and following partner,
- exploratory motion is measured as the number of crossings of the lines marked on the floor of the test box.

Six pairs are used for each dose.

EVALUATIO N

The values of treated partners are compared with the data from 6 pairs of untreated animals using single factor analysis of variance followed by Dunnett's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

In spite of the fact that there may be some analogy between "social anxiety" in humans and the behavior of rats in the social interaction test, there appear some potential complications with this test, such as an increase of social interaction after anxiolytics independent from the environment, dependence on external variables such as time of the day, and the complicated nature of social interaction.

MODIFICATIONS OF THE TEST

Sams-Dodd (1995) described the automation of the social interaction test in rats by a commercially available video-tracking system.

Gheusi et al. (1994) studied the effects of tetrahydroaminoacridine (THA) on social recognition in rats indicating a dissociation of cognitive versus non-cognitive processes.

Doses of 1.0 to 4.0 mg/kg i.p. phencyclidine (PCP) reduce the social interaction time in rats in a dose-dependent fashion. Reversal of the PCP-induced social withdrawal has been used as an animal model for neuroleptic resistant schizophrenia (Carlsson and Carlsson 1990; Corbett et al. 1995).

Olfactory investigation of ovarectomized females by adult male mice decreases during repeated confrontations with the same female intruder, whereas aggressive behavior gradually increases (Winslow and Camacho 1995). Administration of scopolamine blocked decrements in olfactory investigation in repeated confrontations and significantly reduced aggression. Acetylcholinesterase inhibitors enhanced the rate of decrement of olfactory investigation, but had differential effects on aggression.

Wongwitdecha and Marsden (1996) investigated the effects of isolation rearing on anxiety using the social interaction paradigm and compared the effects of diazepam on social interactive behaviors in isolation and socially reared rats.

Sams-Dodd (1997) studied the effect of novel antipsychotic drugs on phencyclidine-induced stereo-typed behaviour and social isolation in the rat social interaction test.

The rat social interaction model has been used by various authors to characterize the potential anxiolytic effects of serotonin receptor antagonists, such as 5-HT_{1C} receptors antagonists (Kennett et al. 1989; Kennett 1992), 5-HT_2 receptor antagonists (Kennett et al. 1994, 1995, 1996a,b, 1997; Costall and Naylor 1995), 5-HT_3 receptor antagonists (Costall et al. 1990; Costall and Naylor 1992; Blackburn et al. 1993), 5-HT_4 receptor antagonists Kennett et al. (1997), cholestystokinin receptor antagonists (Hughes et al. 1990; Costall et al. 1991; Singh et al. 1991) and of nitric oxide synthase inhibitors (Volke et al. 1997).

File and Johnston (1989) reported a lack of effects of 5-HT_3 receptor antagonists in the social interaction test in the rat.

Woodall et al. (1996) described a **competition procedure** in rats. On the first week of experimentation groups of 3 rats (triads) are familiarized with the test box and sweetened milk from a drinking spout located on the end wall. The drinking spout is surrounded by a Perspex tube (4.5 cm diameter) which ensures that only one animal is able to drink at a time. All animals are deprived of water overnight and on the following day placed into the testing box and given access to the sweetened milk for 15 min. On the second week of testing, the rats are not longer water deprived and are given access to the testing box and sweetened milk for 5 min. During the testing period, the rats are observed every 5 s, and a note is made which animal is drinking. This procedure is carried out twice a week for a period of 5 weeks. Drugs are administered to either the dominant or subordinate rat in each triad 15 min prior to testing. Following the drug study, all triads are tested for 2 trials to ensure that their rank orders return to baseline levels. The access of the subordinate member to sweetened milk is increased after administration of an anxiolytic drug.

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E.2.4.4 Elevated plus maze test

PURPOSE AND RATIONALE

Out of many possibilities to modify maze tests (e.g. water maze (Danks et al. 1991), the Y-mace, the radial maze (Di Cicco 1991), and the elevated plus maze (Montgomery 1958; Pellow et al. 1985; Corbett et al. 1991) have found acceptance in many laboratories. The test has been proposed for selective identification of anxiolytic and anxiogenic drugs. Anxiolytic compounds, by decreasing anxiety, increase the open arm exploration time; anxiogenic compounds have the opposite effect.

For effects on learning and memory see Sect. F.3.3.3.

PROCEDURE

The plus-maze consists of two open arms, $50 \times 10 \times 40$ cm, and two enclosed arms, $50 \times 10 \times 40$ cm, with an open roof, arranged so that the two open arms are opposite to each other. The maze is elevated to a height of 50 cm. The rats (200–250 g body weight) are housed in pairs for 10 days prior to testing in the apparatus. During this time the rats are handled by the investigator on alternate days to reduce stress. Groups consist of 6 rats for each dose. Thirty min after i.p. administration of the test drug or the standard, the rat is placed in the center of the maze, facing one of the enclosed arms.

During a 5 min test period the following measures are taken: the number of entries into and time spent in the open and enclosed arms; the total number of arm entries. The procedure is conducted preferably in a sound attenuated room, with observations made from an adjacent room via a remote control TV camera.

EVALUATION

Motor activity and open arm exploratory time are registered. The values of treated groups are expressed as percentage of controls. Benzodiazepines and valproate decrease motor activity and increase open arm exploratory time.

CRITICAL ASSESSMENT OF THE METHOD

The method is rather time consuming, but can be regarded as a reliable measure of anxiolytic activity. Computerized automatic systems are available for elevated plus maze, radial maze, Y-maze, and T-maze (e.g., Technical and Scientific Equipment GmbH, D-61348 Bad Homburg, Germany) and may help to overcome these difficulties.

MODIFICATIONS OF THE METHOD

Latency to enter a mirrored chamber by mice has been described as a behavioral assay for anxiolytic agents (Toubas et al. 1990).

Handley and McBlane (1993) provided an assessment of the elevated X-maze for studying anxiety and anxiety-modulating drugs.

Lapin (1995) studied the effect of handling, sham injection, and intraperitoneal injection of saline on the behavior of mice in an elevated plus-maze. These procedures produce behavior considered to be typical for anxiety inducing drugs. Saline-treated groups taken as controls possess the behavioral profile of stressed and anxious animals.

Pokk et al. (1996) described a method of small platform-induced stress whereby mice were individually placed for 24 h on a small platform (3 cm high, 3.5 cm in diameter) which was fixed at the center of a plastic chamber (20 cm diameter, 40 cm high) and was surrounded by water (1 cm deep) at 22 °C.

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E.2.4.5 Water maze test

PURPOSE AND RATIONALE

Spatial learning of rats can be tested in a water maze as described by Morris (1984) and McNaughton and Morris (1987).

For effects on learning and memory see Sect. F.3.3.5.

PROCEDURE

The water maze consists of a circular tank with 100 cm diameter and a wall 20 cm above the water level. A circular platform (9 cm diameter, covered with white linen material for grip) is hidden 2 cm below the water level. The water is made opaque using titanium dioxide suspension and is kept at about 23 °C during the experiment. Training takes place on three consecutive days, with the rats receiving 4 consecutive trials per day with an inter-trial interval of 6–10 min. Each trial is started from one of four assigned polar positions with a different sequence each day. The latency to find the platform is measured as the time of placement of the rat in the water to the time it finds the platform. If the animal fails to find the platform in any trial within 3 min it is placed on it for 10 s.

EVALUATION

On day four a probe test is performed. The platform is removed and the time spent in the target quadrant (the quadrant in the center of which the platform has been located) and the number of annulus crossings (across the actual location where the platform has been located) in the first 60 s of exposure are measured. The time to the first annulus crossing is also taken as a measure of performance on the 13^{th} (i.e. probe) trial.

Buspirone (Rowan et al. 1990) as well as benzodiazepines (McNaughton and Morris 1987) increase the latency to find the platform in the training period and impair the number and the time of annulus crossings.

CRITICAL ASSESSMENT OF THE METHOD

The water maze test measures learning and memory rather than the anxiolytic activity. If the test is used for memory, rats which have learned quickly and consistently to find the platform are kept in their cages for 2 weeks and then re-tested. Rats which solve the escape immediately, are considered to have retained memory (see also Sect. F.3.3.5).

MODIFICATIONS OF THE METHOD

Bane et al. (1996) used the Morris water maze to study the adverse effects of the non-competitive NMDA receptor antagonist dextromorphan on the spatial learning of rats.

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E.2.4.6 Staircase test

PURPOSE AND RATIONALE

The staircase test for evaluating anxiolytic activity was originally described for rats by Thiebot et al. (1973). When introduced into a novel environment, rodents experience a conflict between anxiety and exploratory behavior manifested by increased vigilance and behavioral activity. In the staircase paradigm, step-climbing is purported to reflect exploratory or locomotor activity, while rearing behavior is an index of anxiety state. The number of rearings and steps climbed are recorded in a 5 min period. The dissociation of these parameters is considered to be characteristic for anxiolytic drugs. The test was modified for rapid screening of anxiolytic activity in mice (Simiand et al. 1984).

PROCEDURE

For experiments with mice the staircase is composed of five identical steps 2.5 cm high, 10 cm wide and 7.5 cm deep. The internal height of the walls is constant along the whole length of the staircase. Naive male mice (Charles River strain) with a weight between 18 and 24 g are used. Each animal is used only once. The drug or the standard is administered orally 1 h or 30 min subcutaneously before the test. The animal is placed on the floor of the box with its back to the staircase. The number of steps climbed and the number of rears are counted over a 3-min period. A step is considered to be climbed only if the mouse has placed all four paws on the step. In order to simplify the observation, the number of steps descended is not taken into account. After each test, the box has to be cleaned in order to eliminate any olfactory cues which might modify the behavior of the next animal.

EVALUATION

Twelve mice are used for the untreated control group, each drug group, and for the group receiving the standard. The average number of steps and rearings of the control group is taken as 100%. The values of treated animals are expressed as percentage of the controls.

CRITICAL ASSESSMENT OF THE METHOD

The staircase test has been proven as a simple and reliable method for screening of anxiolytics in several laboratories. Many applications and modifications have been described in the literature (Houri 1985; Steru et al. 1987; Keane et al. 1988; Emmannouil and Quock 1990; Simiand et al. 1993).

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E.2.4.7 Cork gnawing test in the rat

PURPOSE AND RATIONALE

Cork gnawing behavior in the rat has been proposed as a screening method for buspirone-like anxiolytics by Pollard and Howard (1991).

PROCEDURE

Adult male Evans rats serve as subjects. They are housed 4 per cage on a regular light/dark cycle with free access to food and water except for the period between injection and the end of a test session. For the test session one animal is placed in a stainless steel cage with wire mesh bottom. A session consists of placing the subject in the test cage with a cork stopper weighing between 2–3 g for 30 min. Initially, the amount gnawed is relatively high and variable within and between subjects. After 30 training sessions, the amount is low and stabilized. The test compounds are injected 30 min before the test and food is withdrawn.

EVALUATION

Each cork is weighed to the nearest 0.01 g before and after the session. The average cork loss during the previous control days is taken as baseline and the amount after drug treatment is expressed as percentage of baseline. Buspirone-related compounds as well as benzodiazepines and meprobamate show a dose dependent increase of cork gnawing, but amphetamine, chlorpromazine, imipramine and morphine do not.

CRITICAL ASSESSMENT OF THE METHOD

The test is worthwhile to be mentioned, since buspirone – whose anxiolytic action was discovered during clinical trials to assess possible antipsychotic action and not by use of animal tests for anxiolysis – is active in this test but not in most other classical tests for anxiolytic activity.

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E.2.4.8 Distress vocalization in rat pups

PURPOSE AND RATIONALE

Measurement of ultrasonic vocalization induced by tail-holding in rat pups was proposed as a simple screening method for anxiolytic drugs by Gardner (1985).

PROCEDURE

Wistar rat pups are bred on site and left undisturbed with their mother, except for cage-bedding replacement, until the day of testing. The pups are tested at 9–12 days of age. On the day of testing the pups are separated from their mother and taken in their home cage to the quiet experimental room. In the morning all pups are subjected to handling stress and the magnitude of their ultrasound emission is observed. The stress consists of holding the pup by the base of the tail, between forefinger and thumb of the experimenter, and thus suspending it 5 cm above the bench for 30 s. A prior control recording (30 s) is taken when the pup is held gently in the experimenter's hand, whereby the pups emit only a few ultrasounds. Responses when held by the tail are more than 10 times higher. This entire hand-holding-tail-holding procedure is immediately repeated.

Ultrasounds are recorded with suitable detectors with 42 kHz as the center of a 10 kHz recording range. The output of the detectors is fed into pen recorders. The total number of ultrasonic cries in the two sessions of hand holding and the two sessions of tail holding are calculated and used as the control activity of each pup. Any pup producing a total of less than 50 ultrasounds when held by the tail is excluded from the drug study. The pups are kept in the home cage in the test laboratory until the afternoon. Three to four hours after the first test the pups are randomly allocated to several equally seized groups, weighed, marked, and dosed intraperitoneally either with the vehicle or drug and placed back in the home cage. Thirty min after dosing, each pup is subjected to the same handling stress as that used in the morning session, and the total number of sounds produced is calculated in the same way.

EVALUATION

The afternoon response to tail holding is expressed as a factor of the morning response. The mean factor for the saline-treated animals is taken to be 100% in calculations of percentage changes in ultrasound emission by drugs.

CRITICAL ASSESSMENT OF THE METHOD

Anxiolytic benzodiazepines dose-dependent inhibit vocalization. Amitriptyline and haloperidol have no effect. Chlorpromazine, muscimol and prazosin reduce sound at doses which also induce overt sedation. Therefore, the method can be regarded as relatively specific for anxiolytic activity.

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E.2.4.9 Schedule induced polydipsia in rats

PURPOSE AND RATIONALE

Food deprived rats exposed to a procedure in which food is delivered intermittently will drink large amounts of water if given the opportunity to do so. This behavioral phenomenon is termed schedule-induced polydipsia and is an example of a more general class of behaviors termed adjunctive behaviors (Falk 1971; Pellon and Blackman 1992). Adjunctive behaviors have been cited as potential animal models of human obsessive-compulsive disorders (Pitman 1989).

PROCEDURE

Male Wistar rats weighing 180–250 g are individually housed at a 12 h/12 h light/dark cycle for a 1 week acclimation period with free access to food and water. Then they are placed on a restricted diet which maintains 80% of their free feeding body weight. To induce polydipsia, rats are placed in test chambers housed in sound attenuated boxes where a pellet dispenser automatically dispenses two 45 mg pellets on a fixed time 60-s (FT-60s) feeding schedule over a 150 min test session. Water is available at all times in the test chambers. After 4 weeks exposure to the FT-60s feeding schedule, approximately 80% of the rats meet the pre-determined criterion for water consumption (greater than 60 ml water per session) and are considered to have polydipsic behavior.

Rats receive the test compounds in various doses daily or the vehicle intraperitoneally 60 min prior to testing. They are tested once a week to assess schedule induced polydipsia. Water bottles are weighed before and after the 150-min test sessions.

EVALUATION

The experimental data comparing the effects of chronic administration of compounds on schedule-induced polydipsia are analyzed with the Mann Whitney *U*-test.

MODIFICATIONS OF THE METHOD

Yadin et al. (1991) proposed spontaneous alternation behavior in rats as an animal model for obsessive-compulsive disorder. Food-deprived rats were run on a T-maze in which both a white and a black goal box were equally baited with a small amount of chocolate milk. Each rat was given 7 trials every other day during which it was placed in the start box and allowed to make a choice. The mean number of choices until an alternation occurred was recorded. After a baseline of spontaneous alternation was achieved the rats were treated with the non-selective serotonin agonist

5-methoxy-N,N-dimethyltryptamine (5-MeODMT) (1.25 mg/kg i.p.) or the selective 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide (8-OH-DPAT) (2 mg/kg i.p.) which both disrupted the spontaneous alternation. A course of chronic treatment (2 × 5 mg/kg for 21 days) with the selective 5-HT uptake blocking agent fluoxetine had a protective effect on the 5-MeODMT-induced disruption of spontaneous alternation behavior. The authors speculated that serotonergic manipulations of spontaneous alternation may be a simple animal model for the perseverative symptoms or indecisiveness seen in patients with obsessive-compulsive disorder.

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E.2.4.10 Four plate test in mice

PURPOSE AND RATIONALE

The four plate test in mice has been described by Aron et al. (1971), Boissier et al. (1968) as a method for the rapid screening of minor tranquilizers.

PROCEDURE

The test box has the shape of a rectangle (25 × 18 × 16 cm). The floor is covered with 4 identical rectangular metal plates (8 × 11 cm) separated from one another by a gap of 4 mm. The plates are connected to a source of continuous current which applies to 2 adjacent plates a mild electrical shock of 0.35 mA for 0.5 s. This evokes a clear flight reaction of the animals.

Adult male Swiss albino mice, weighing 17 to 23 g, are randomly divided into different groups. Thirty min before the test the animals are injected intraperitoneally with the test drug or the vehicle.

At the beginning of the test, the mouse is gently dropped onto a plate and is allowed to explore the enclosure for 15 s. After this, every time the animal crosses from one plate to another, the experimenter electrifies the whole floor for 0.5 s, which evokes a clear flight-reaction of the mouse which often crosses 2 or 3 plates. If it continues running, no new shock is delivered during the following 3 min.

EVALUATION

The number of times the apparatus is electrified is counted each minute for 10 min. The delivery of shocks decreases dramatically the motor activity. The number of shocks received during the first min is taken as parameter. This number is increased by minor tranquilizers, such as benzodiazepines, but not by neuroleptics and psychoanaleptics.

CRITICAL ASSESSMENT OF THE METHOD

The test is of value to differentiate minor tranquilizers, such as benzodiazepine anxiolytics, from neuroleptics. However, some stimulants (e.g. amphetamine) produce an increase in punished plate crossings and some anxiolytics do not.

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E.2.4.11 Footshock induced freezing behavior in rats

PURPOSE AND RATIONALE

Footshock-induced freezing behavior in rats has been proposed as a model for anxiolytics by Conti et al. (1990).

PROCEDURE

Male Sprague-Dawley rats with a weight between 200 and 350 g are used. The animals receive a single i.p. injection of the test compound or the vehicle 30 min prior to being placed in a standard conditioning chamber (e.g., Coulborn Instruments) for a 6.5 min session. Two and 2.5 min after the start of the session, a scrambled footshock (0.5 mA, 0.5 s) is delivered through the grid floor of the chamber. Using an assembly of push buttons interfaced with a computer, an observer monitors the amount of time each animal spends engaged in the following mutually exclusive behaviors:

- Freezing: immobility with rigid body posture
- Sedated posture: sitting or sleeping
- Small exploratory movements: movements involving the torso or front paws only, vertical movements of the head, or sniffing.
- Locomotion: activity involving hind paws, grooming or rearing.

Frequency of rearing is also counted. All behaviors are monitored for the entire 6.5 min session.

EVALUATION

Duration of foot-shock induced freezing after the second shock is taken as the critical parameter. Time spent in freezing posture after administration of test compounds is compared with the controls. Anxiolytics like diazepam and buspirone show dose-dependent effects, but not haloperidol.

CRITICAL ASSESSMENT OF THE METHOD

The method seems to discriminate anxiolytics including buspirone from other centrally acting drugs.

MODIFICATIONS OF THE METHOD

Footshock-induced ultrasonic vocalization has been suggested as another model of anxiety (Tonoue et al. 1986; Kaltwasser 1990; Miczek et al. 1991; De Vry et al. 1993; Nielsen and Sánchez 1995; Schreiber et al. 1998). Test cages ($22 \times 22 \times 22$ cm) made of grey Perspex are equipped with a metal grid floor (distance between the bars = 1 cm). Electric footshocks are delivered from a 12 bit programmable shock source and ultrasounds are picked up by a microphone (range 18–26 Hz) placed in the center of the cage lid. The total vocalization time is measured. The rats are placed individually in the test cages and immediately receive a series of 0.5 mA inescapable footshocks each of 1 s duration with a shock interval of 5 s. The vocalization is measured for a 10 min period starting 1 min after the last shock. Drugs or saline are given subcutaneously 30 min before the test.

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E.2.4.12 Experimental anxiety in mice

PURPOSE AND RATIONALE

Ogawa et al. (1966, 1990, 1993) designed a communication box to induce experimental anxiety in mice by employing intraspecies emotional communication. The inside of the communication box was divided into foot-shock and non-foot-shock compartments by transparent plastic boards. The animals, which were individually placed into each compartment, were unable to make physical contact with one another, but were able to receive other cues such as visual, auditory and olfactory sensations. During the foot shock period, the animals placed in the non-foot-shock compartments were exposed to the emotional cues from foot-shocked animals, such as shrieks, smell of feces or urine, and jumping response.

PROCEDURE

The floor of the communication box is equipped with grids for electric shock. The inside is divided into small compartments (10×10 cm), consisting of foot-shock compartments with a grid floor and non-foot-shock compartments with a grid floor covered by transparent plastic boards. The foot-shock compartments are arranged such as to surround the non-foot shock compartments.

The experimental groups consist of the following 3 groups: sender group, responder group, and food-yoked group to responder. Sender animals receive a foot shock of 10-s duration at intervals of 50 s for 3 h. The

electric current for the shock is increased step-wise from 1.6 mA to 2.0 mA at a rate of 0.2 mA per 1 h. Responders are exposed daily to the emotional responses of sender animals, 3 h per day for 3 days. Sender animals are changed daily to naive mice to prevent a reduced emotional response to foot shock based on adaptation or learned helplessness due to repeated exposure. Both sender and responder animals are placed individually in each compartment of the communication box 15 min before beginning of the shock period. On day-1, responder animals are returned to their home cages after the 3-h foot shock period. An day-2, after completing the foot-shock period, they are transferred to metal cages and are housed in the cages with 4 animals per cage under food-deprivation condition. Food-yoked control animals are maintained to the metal cage during the foot-shock period under the aggregated housing condition (5 animals each) and then they are returned to the home cages after the foot shock period. From beginning of the day-2 experiment to completion of the day-3 experiment, they are maintained in the metal cages under aggregating housing. On day-3, just after completing the foot-shock period, the responders are sacrificed by chloroform, and their stomachs are removed. The stomachs are visually inspected for lesions.

Drugs are administered orally at different doses either with a single dose on day-3 or daily 30 min before the shock period.

EVALUATION

Data are reported as the incidence of mice with gastric lesions characterized by slight erosions or bleeding. Active anxiolytics reduce the incidence of gastric ulcers found in food-deprived animals. The incidence in food-yoked animals is much lower. The data are analyzed by Fisher's exact probability test.

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E.2.4.13 mCPP induced anxiety in rats

PURPOSE AND RATIONALE

The metabolite of the antidepressant drug trazodone 1-(3-chlorphenyl)piperazine (= mCPP), classified as 5-HT_{1C} agonist (Rocha et al. 1993; Gibson et al. 1996) or 5-$HT_{1B/2C}$ agonist (Dryden et al. 1996), has been shown to be anxiogenic both in man and in rats (Curzon et al. 1991). The compound induces hypophagia (Samanin et al. 1979; Dryden et al. 1996; Yamada et al. 1996) and hypolocomotion (Kennett et al. 1996, 1997a), inhibits social interaction in rats, diminishes exploratory activity of rats in the open field test (Czyrak et al. 1994; Meert et al. 1997) and in the light-dark box test (Bilkei-Gorzo et al. 1998), induces hyperthermia (Aulakh et al. 1995; Kennett et al. 1997b) and reduces ultrasound-induced defensive behavior in the rat (Beckett et al. (1996). Antagonism against these symptoms has been proposed as a screening model for anxiolytic drugs (Bilkei-Gorzo et al. 1996, 1998; Wallis and Lal 1998).

PROCEDURE

Male Sprague Dawley rats (220–250 g) are housed in groups of 6 under a 12 h light/dark cycle with free access to food and water.

mCPP-induced locomotion

Rats are placed in a room adjacent top the experimental room on the day of the procedure. They are dosed either orally 1 h, or i.p. 30 min before the locomotion test with test compound or vehicle, and injected 20 min before the test with 7 mg/kg mCPP i.p. or saline in groups of four. Rats are returned to their home cages after dosing. At 0 h they are each placed in automated locomotor activity cages made of black Perspex with a clear Perspex lid and sawdust covered floor under red light for 10 min. During this time, locomotion is recorded by means of alternately breaking two photocell beams traversing opposite ends of the box 3.9 cm above floor level.

mCPP-induced hypophagia

Rats are individually housed on day 1 and on day 3 they are deprived of food. Twenty-three hours later, the are orally treated with the test drug or vehicle and returned to their home cages. Forty min later, they are given 5 mg/kg mCPP or saline i.p. and again returned to their home cages. After a further 20 min, weighted amounts of their normal food pellets are placed in their food hoppers and the amount remaining after 1 h is measured.

EVALUATION

The effect of the test compound on mCPP-induced hypolocomotion is determined by one-way ANOVA and Newman-Keuls test. The dose producing 50% disinhibition of mCPP is also estimated. Feeding test data are subjected to one-way ANOVA and Dunnett's test.

MODIFICATIONS OF THE METHOD

Griebel et al. (1991) described neophobic and anxious behavior in mice induced by m-CPP.

Czyrak et al. (1994) measured the antagonism of anti-psychotics against the mCPP-induced hypothermia in mice.

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E.2.4.14 Acoustic startle response in rats

PURPOSE AND RATIONALE

The acoustic startle reflex is a relatively simple behavior that occurs naturally in mammals and is affected by a variety of treatments. It consists of a series of rapid movements beginning at the head and moving caudally involving contraction and extension of major muscle groups in response to auditory stimuli with a rapid onset, or rise time. Responses are graded in amplitude in relation to stimulus intensity, and may show habituation and sensitization. Startle response can be used to determine sites and mechanisms of drug action (Davis 1982).

PROCEDURE

Male Wistar rats weighing about 200 g are used. Acoustic startle reflexes are measured in a specially build apparatus, e.g., Coulborn Instruments Acoustic Response Test System. The animals are individually placed in $8 \times 8 \times 16$ cm open air cages that restrict locomotion but do not immobilize the animal, and are placed on one of four platforms within a sound-attenuating acoustic chamber. A ventilating fan provides an ambient noise level. Acoustic stimuli consist of white noise bursts lasting 20 ms at 98 dB and 124 dB SPL. Simultaneously with the rapid onset of each stimulus, the animal's physical movement within the cage on the platform is measured for 200 ms as an electrical voltage change via a strain gauge which is converted to grams of weight change following analog to digital conversion. Data are recorded automatically by an interfaced microcomputer.

Pre-tests are performed with all animals to obtain control values. The animals are treated 2 h prior the experiment with test drugs or vehicle given orally or subcutaneously.

EVALUATION

The results are given as percentage of the change, related to the values obtained in the pre-test and assessed by a one-way ANOVA, followed by Dunnett's test when appropriate.

MODIFICATIONS OF THE METHOD

The test has been modified in various ways, e.g., inhibition by a prepulse (Keith et al. 1991; Rigdon and Viik 1991; Taylor et al. 1995) or fear-induced potentiation (Davis 1986, 1992).

Sipes and Geyer (1995) studied the disruption of prepulse inhibition of the startle response in the rat by DOI (2,5-dimethoxy-4-iodophenylisopropylamine) which is mediated by 5-HT_{2A} receptors. The authors suggested that studies of the serotonergic substrates of prepulse inhibition may provide a model of the possible serotonergic role in the sensorimotor gating abnormalities in schizophrenia and obsessive compulsive disorder patients.

Schulz et al. (1996) performed acoustic startle experiments in rats with a potent and selective non-peptide antagonist of the corticotropin releasing factor receptors.

Walker and Davis (1997) found that the amplitude of acoustic startle response in rats was increased by high illumination levels.

Devices to register the intensity of fear-potentiated startle response in rats were described by Hijzen et al. (1995).

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E.2.4.15 Unconditioned conflict procedure (Vogel test)

PURPOSE AND RATIONALE

Vogel et al. (1971) described a simple and reliable conflict procedure for testing anti-anxiety agents. Thirsty, naive rats were administered shocks while licking water.

PROCEDURE

The apparatus is a clear Plexiglas box (38 × 38 cm) with a black Plexiglas compartment (10 × 10.5 cm) attached to one wall and an opening from the large box to the small compartment. The entire apparatus has a stainless-steel grid floor. A water bottle with a metal drinking tube is fitted to the outside of the small compartment, so that the tube extended into the box at a height 3 cm above the grid. Rats lick in bursts with a relatively constant rate of 7 licks per sec. A drinkometer circuit is connected between the drinking tube and the grid floor of the apparatus, so that the rat completes the circuit whenever it licks the tube. Shock is administered to the feet of the animal by switching the connections to the drinking tube and grids from the drinkometer to a shocker which applies an unscrambled shock between the drinking tube and the grid floor.

Naive adult male rats are used. Thirty min after intraperitoneal injection, the rat is placed in the apparatus and allowed to find the drinking tube and to complete 20 licks before shock (available at the tube for 2 s) is administered. The rat controls shock duration by withdrawing from the tube. A 3-min timer is automatically started after the termination of the first shock. During the 3-min period, shocks are delivered following each twentieth lick. The number of shocks delivered during the 3-min session is recorded for each animal.

EVALUATION

The number of shocks received after treatment is compared with untreated animals. Benzodiazepines increase dose-dependent the number of shocks. Barbiturates in low doses and meprobamate, but not d-amphetamine or scopolamine, are active in this test.

CRITICAL ASSESSMENT OF THE METHOD

The method is far more simple and less time consuming than the methods using conflict behavior after intensive training. The specificity may be less than that of the Geller paradigm.

MODIFICATIONS OF THE METHOD

The method and the apparatus have been slightly modified by Patel and Malick (1982), Patel et al. (1983) and Sanger et al. (1985).

Miklya and Knoll (1988) showed an increase of sensitivity of the method using rats deprived of food but supplied with tap water ad libitum for 96 h, than fed with dry pellets and punished for drinking during feeding. The punished drinking test has not only be used for identifying and studying anxiolytic agents, but also as a method for measuring anxiogenic activity (Uyeno et al. 1990).

La Marca and Dunn (1994) studied α_2-antagonists after intravenous administration in the Vogel lick-shock conflict paradigm.

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E.2.4.16 Novelty-suppressed feeding

PURPOSE AND RATIONALE

Placing a hungry rat into an unfamiliar environment with access to food results in a suppression of feeding behavior relative to the condition when the test environment is familiar. This effect has been termed hyponeophagia (Shephard and Broadhurst 1982) and occurs because of the novelty of the test environment. The avoidance of novel foods is termed food neophobia. Both hyponeophagia and food neophobia have been assumed to measure emotionality or anxiety by eliciting a conflict situation arising from a fear of the novel setting and foods, and the drive to eat (Porschel 1971). A number of investigators have adopted these paradigms to explore the behavioral effects of anxiolytics (Soubrie et al. (1975;Cooper and Crummy 1978; Borsini et al. 1993).

PROCEDURE

The testing apparatus consists of individual Plexiglas open fields, 76 × 76 × 46 cm. Thirty Purina lab chow pellets are placed in a pile directly in the center of the open field.

Animals are handled for 3 weeks prior the behavioral testing. Forty-eight hours prior to testing, all food is removed from the home cage, although water is still

available ad lib. One h prior to testing, animals receive an intraperitoneal injection of test drugs or vehicle. At the time of testing, the animals are placed into individual open fields containing the food, and the latency to begin eating is measured. If the animal has not eaten within 720 s, the test is terminated and the animal is assigned a latency score of 720 s.

EVALUATION

The data are analyzed by a one-way analysis of variance followed by Fisher Last Significant Difference post hoc tests. An anxiolytic effect is defined as a significant decrease in mean latency to begin eating compared with vehicle controls.

CRITICAL ASSESSMENT OF THE METHOD

The test has the advantage of simplicity for screening procedures.

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E.2.4.17 Shock probe conflict procedure

PURPOSE AND RATIONALE

The shock probe conflict procedure, an assay responsive to benzodiazepines, barbiturates and related compounds, was described by Meert and Colpaert (1986). Rats being placed in a novel test environment containing a probe, explore the environment and also the probe. The exploration of the probe, quantified as the number of times that the animal makes physical contact with it, is reduced when the probe is electrified. Rats treated with anxiolytics continue to touch the electrified probe.

PROCEDURE

Apparatus: The test environment consists of a Plexiglas chamber, measuring $40 \times 40 \times 40$ cm, and having a metal grid floor. A Teflon probe (∅: 1 cm) with two uninsulated wires (∅: 0.5 mm) each independently wrapped 25 times around it, is inserted from the front panel protruding for a length of 6.5 cm into the test box, 3 cm above the floor of the chamber. The wires are connected to a shocker. Whenever the animal touches both wires simultaneously with some part of its body, a DC current flows through the animal. At the same time, a counter is triggered. Normally, a shock intensity of 0.9 mA is used.

Sixty min after treatment with saline or test substance, the animal is placed in a back corner of the testbox facing away from the probe. The test session starts from the moment makes the first contact and receives the first shock. The number of responses the animal makes during the subsequent 5-min episode is counted.

EVALUATION

Dose-response curves can be established for various drugs at different shock intensities. The Mann-Whitney *U*-test is used to evaluate differences between experimental conditions. To control whether a drug treatment increases responding above the saline control level, an one-tail *t*-test is used; a two-tail test in other cases.

CRITICAL ASSESSMENT OF THE METHOD

The procedure requires neither behavioral training nor expensive equipment and overcomes some of the limitations that are typical for other conflict procedures. However, the procedure still uses electric shock as inhibitory stimulus.

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E.2.4.18 Ultrasound induced defensive behavior in rats

PURPOSE AND RATIONALE

Rats exposed to aversive stimuli display specific defence behavior as a part of their natural survival strategy. One component of this behavior is the production of ultra-

sonic calls in the 20–27 kHz range, which are thought to serve a communication role. Artificially gen-erated ultrasound produces intensity-related locomotion, characteristic of defensive behavior (Beckett et al. 1996).

PROCEDURE

The apparatus (Beckett and Marsden 1995) consists of a circular open field arena, 75 cm in diameter, 46 cm high walls, with a video camera suspended above. Locomotor behaviors are recorded and analyzed using a computer automated tracking system capable of following rapid movements (VideoTrack, CPL Systems, Cambridge, UK). This allows the ultrasound-induced change in locomotor behavior to be quantified in maximum speed, average speed and distance traveled by the animals. Data are expressed as 15 sequential 20-s bins over the duration of the experiment.

Ultrasound (continuous tone, square wave, 20 kHz) is produced using a multifunction signal generator at sound pressure intensities of 65, 72 and 75 dB, as measured from the arena, 20 cm horizontally from the speaker. Sound is delivered to the testing arena via a high frequency piezo electric speaker mounted at a height of 40 cm on the wall of the testing arena. The signal frequency and intensity delivered to the speaker are monitored using a digital oscilloscope. White noise is generated using a standard generator and the sound intensity measured as above.

Animals are placed in the test arena 20 min after intraperitoneal injection of drug or vehicle and locomotor activity is measured. After 2 min they are exposed to a 1-min, 20 kHz, square wave ultrasound tone (65, 72 or 75 dB sound pressure intensity, randomized) followed by a further 2 min without sound. This procedure is repeated for each intensity with a 1-min inter-procedure interval. Locomotor activity values are then calculated for the maximum speed, average speed and total distance traveled throughout the 5-min test period and expressed as a series of 15–20-s time epochs.

EVALUATION

Maximum speed is analyzed using a two-way ANOVA. Significant interactions between treatment and time are followed by one-way ANOVAs for individual time points with post-hoc Duncan's new multiple range test.

MODIFICATIONS OF THE METHOD

Moleweijk et al. (1995) evaluated ultrasound vocalizations of adult male rats in association with aversive stimulation as a screening method for anxiolytic drugs.

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E.2.4.19 Anxiety/defense test battery in rats

PURPOSE AND RATIONALE

Blanchard et al. (1989, 1990, 1992) described a set of procedures designed to assess the defensive reactions of rats to a natural predator, the cat. These tests involve a brief confrontation of laboratory rats with an unconditioned threat stimulus (cat) which, to preclude physical contact, is presented behind a wire mesh barrier. The primary measures, taken both during and after cat presentation, include movement arrest and risk assessment (proxemics/activity test) and the inhibition of non-defensive behaviors (eat/drink or freezing test).

PROCEDURE

The test apparatus for both the proxemics/activity and eat/drink procedures consists of two parallel subject chambers ($53 \times 20 \times 25$ cm). The inside walls of each chamber are constructed of opaque black Plexiglas, while outer walls and lids are clear Plexiglas to allow video recording from lateral and overhead views. The end wall of each chamber, constructed of wire mesh, adjoins a separate cat compartment. Subject movements are monitored by five photocells mounted at equal distances over the length of each chamber, and a food hopper and drinkometer are positioned 2.5 cm to each side of the central photocell. Access to the food hopper/drinkometer can be prevented by insertion of Plexiglas gates.

Each rat (Long-Evans strain, female or male, about 100 days old) receives the same injection (drug or saline) in each of the two successive paradigms. The initial study assesses the effects of cat exposure on proxemics/activity, followed 7 days later by analysis of eat/drink behavior during and after cat exposure. Both procedures are carried out under dim red light.

Proxemic/activity testing. Rats are individually placed in each compartment of the test apparatus. Following a 5-min pre-cat period, the cat is introduced to the cat compartment for 5 min. Following removal of the cat, behavior is recorded for a further 15 min post-cat period, for which measures are summed in three, 5-min blocks. The test session is video recorded for analysis of lying, crouching, rearing, locomotion and grooming. Proxemic location is measured by a digitizing sys-

tem which divides the length of the subject compartment into thirds, indicating animal's location near the cat compartment, in the midsection of the box, or far from the cat compartment. Assessment of transits indicate movement from one section to another.

Eat/drink testing. Rats are individually given 2 g of finely crushed chocolate cereal on the 2 days after the proxemic/activity test, to familiarize them with this highly preferred food. In order to induce a mild water deprivation, water bottles are removed, 24 h prior to eat/drink testing. On the test day, animals are individually placed in the subject compartments for a 5-min pre-cat period, during which the food hopper and water dispenser are concealed with Plexiglas gates. At the beginning of the 5-min cat period, these gates are removed allowing free access to the crushed chocolate cereal and water. After removal of the cat, the rats are monitored for a further 15-min post-cat period. Measures of eating frequency and duration, and drinking frequency are taken for the cat and post-cat periods.

EVALUATION

The data are analyzed by analysis of variance (ANOVA). Subsequent comparisons between treatment groups and control are carried out using Newman-Keuls procedures.

MODIFICATIONS OF THE TEST

Blanchard et al. (1986a,b, 1989) developed a battery of tests designed to elicit a wide range of active and passive defensive activities in *wild rats*.

Griebel et al. (1997, 1998a,b) designed a mouse defense test battery in which Swiss mice were confronted with a natural threat (a rat) and behaviors associated with this threat were recorded.

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E.2.4.20 Marmoset human threat test

PURPOSE AND RATIONALE

The behavior of the common marmoset (*Callithrix jacchus*) as described by Stevenson and Poole (1976) can be used for evaluation of potential anxiolytic drugs (Costall et al. 1988).

PROCEDURE

Male or female laboratory bred common marmosets weighing 350–400 g are housed in single sex pairs. Holding rooms are maintained at 25 °C at a humidity of 55% on a 12 h light/dark cycle. Tests are conducted between 13:30 and 15:30 in the normal holding room. The holding cages are 75 cm high, 50 cm wide and 60 cm deep.

A behavioral change characterized by retreat from, and posturing toward a human threat is initiated by a human observer standing in close proximity in front of the holding cage. Changed behavior is recorded over a 2 min period by the observer. The behavioral measures selected are:

i the % of time spent on the cage front in direct confrontation with the human threat,
ii the number of body postures, primarily shown as raising the tail to expose the genital region with varying degrees of piloerection, anal scent marking and slit stare with flattened ear tufts.

The animals are used at 7 day intervals and are subject to a random cross-over of treatments.

Drugs are administered 45 min before exposure to the human threat situation.

EVALUATION

Statistical analysis is performed with one-way analysis of variance followed by Dunnett's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The human threat test in marmosets has the advantage of using primates instead of rodents. However, it is subject to individual scoring of the observer.

MODIFICATIONS OF THE TEST

Borsini et al. (1993) used female **cynomolgus monkeys** to test aggressiveness against the observer.

Cilia and Piper (1997) developed a method of measuring conspecific confrontation-induced behavioral changes in common marmosets together with automated monitoring of locomotor activity as a possible model of anxiety.

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E.2.4.21 Aversive brain stimulation

PURPOSE AND RATIONALE

Electrical stimulation of brain aversive areas, in particular the midbrain central gray, induces defensive reaction and/or flight behavior in several species and, therefore, may be viewed as an animal model of anxiety or of panic attack. Most studies used intracerebral microinjections of neurotransmitters, their agonists and antagonists to elucidate the mechanisms of aversive or antiaversive effects (Schütz et al. 1985; Graeff et al. 1986, 1990, 1991 1993, 1997; Audi et al. 1988, 1991; Brandão et al. 1991, 1993; Broekkamp et al. 1991; Nogueira and Graeff 1991, 1995; Motta and Brandão 1993; Aguiar and Brandão 1994, 1996; Melo and Brandão 1995; Motta et al. 1995; De Araujo et al. 1998). Other groups evaluated the effect of drugs, e.g., serotonin receptor antagonists, on periaqueductal gray stimulation induced aversion after peripheral application (Bovier et al. 1982; Clarke and File 1982; Jenck et al. 1989, 1996, 1998; Beckett and Marsden 1997).

PROCEDURE

Surgery. A stainless steel bipolar twisted electrode, insulated to the tip which is cut square to the shaft, is implanted to the dorsal part of the periaqueductal gray matter of male Wistar rats weighing 370–450 g under pentobarbital anesthesia. According to the atlas of Paxinos and Watson (1982), the coordinates for the electrode tip are 5.8 mm posterior to the bregma, 0.2 mm lateral to the midline and 5.0 mm ventral to the surface of the skull. The electrode is held in place with dental cement and five screws threaded into the skull.

Behavioral procedure. Animals are placed in a rectangular cage ($20 \times 36 \times 20$ cm high) with a grid floor and a 2 cm high barrier dividing the cage in half. They are allowed to explore freely for 10–15 min before the stimulation begins.

Strain stimulation consists of constant current square wave, 0.1 ms duration, monophasic pulses conducted from the neurostimulator (Grass S88 + stimulus isolation unit SIU8) to the electrode by way of flexible wire leads. Pulse duration, pulse frequency and stimulation intensity are monitored by an oscilloscope. Animals are screened for stimulation-induced aversion using a fixed stimulation frequency of 50 Hz; current is raised slowly until aversive behavioral signs are observed. Aversive effects are first characterized by visible autonomic reactions (increase in respiratory rates, piloerection, eventually mydriasis) in animals which are behaviorally frozen. Increasing the intensity induces, following a freezing period, active behavioral signs, ranging from ear dressing and head weaving to sudden running and attempts to escape out of the cage.

With this fixed stimulation intensity, aversive behavior is shaped into an operant escape response: rats are trained to stop the stimulation by escaping from one compartment to the opposite compartment of the cage. Brain stimulation is switched off when the rat crosses the middle line separating the two compartments or after a maximal cut-off time of 20 s. A trial is applied every min. Three to 10 daily sessions of 30 trials are required to obtain stable responses.

Once an animal displays steady performances during 3 consecutive days on this task with fixed intensity and fixed stimulation frequency, it undergoes the next step of training aimed at determining its stimulation frequency threshold for escape reaction. This consists of testing the animals in a threshold procedure in which the frequency is varied while stimulation inten-

sity is held constant. This procedure keeps the size of the stimulation field around the electrode tip constant.

A stimulation intensity is chosen and defined as the threshold intensity eliciting escape when the stimulation frequency is 50 Hz. With this intensity held fixed, a method of limits is employed to determine the frequency threshold for escape: the stimulation frequency is either decreased or increased depending on the response displayed by the animal on the previous trial. Starting from 50 Hz, stimulation frequency is decreased by 5 Hz steps following a trial in which the rat responds to the stimulation, and is increased by 5 Hz steps, in case the animal fails to respond to the stimulation. When a response is made, the time elapsing between the onset of the stimulation and the moment the animal crosses the midline barrier is also recorded as escape latency; no response is associated with the maximum cut-off time of 20 s.

Frequency threshold is calculated as the average frequency eliciting an escape reaction during a 20-min pre-injection session; an average escape latency is calculated the same way. Drugs are then injected intraperitoneally at various doses and 35 min following administration, frequency threshold is determined again over a 25–30 min post-injection session. Drug effects can be estimated by comparing thresholds and latencies for each animal before and after injection. Doses are injected at least 4 days in a counterbalanced order. Animals serve as their own controls and can undergo several treatments.

EVALUATION

Data are analyzed by means of analysis of variance, followed by paired *t*-tests. Dose-response curves are established for active drugs.

MODIFICATIONS OF THE METHOD

The group of Graeff and Brandão (1986) used an electrode-cannula, called chemitrode, for electrical stimulation and microinjection of drugs or neurotransmitters at the same place of the periaqueductal gray. This chemitrode was made of stainless steel cannula (outside diameter 0.6 mm, length 12.5 mm) glued to a brain electrode made of stainless steel (diameter 250 mm) enamel insulated except at the cross-section of the tip, reaching 1 mm below the lower end of the cannula (Nogueira and Graeff 1995).

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E.2.5 Conditioned behavioral responses

E.2.5.1 Sidman avoidance paradigm

PURPOSE AND RATIONALE

Sidman (1953) described an apparatus for the evaluation of two temporal parameters (shock-shock interval and response-shock interval) of the maintenance of avoidance behavior by the white rat. The procedure has been widely used to evaluate CNS depressant compounds, neuroleptics, anxiolytics and sedatives.

PROCEDURE

The test cage is equipped with a single lever and a light. This cage is enclosed in a sound-attenuating chamber with a fan and with a speaker emitting a white-noise auditory background. The test cage has a grid floor of steel bars which are attached to a scrambled shock source. The data are recorded in an adjacent room. Male Sprague-Dawley rats with a starting weight of 250 to 300 g are housed in individual cages. They are trained to avoid an unsignalled shock by repetitive lever-pressing responses. A shock (1.5 mA for 0.5 s) is delivered to the grid floor every 15 s if no responses occur (shock-shock interval of 15 s = SS-15 s). A lever press (response) will delay the oncoming shock for 30 s (response-shock interval of 30 s = RS-30 s). The responses do not accumulate for delays of shock; a shock will be delivered 30 s after the last response is made even if 10 responses are made 31 s prior. Every 30 min, the total number of shocks received and the total number of responses made are accumulated and constitute the basic data. The animals are trained until they maintain a stable response rate and receive no more than 100 shocks/five hour test session. After reaching these criteria of performance, experimental compounds are administered and their effects on the performance of this learned avoidance behavior are evaluated. The experimental compounds or the standard are usually administered by i.p. injection immediately prior to testing in volumes of 1 ml/kg of body weight. Depressant drugs lower the rate of lever presses and increase the number of shocks received. Stimulant drugs increase the rate of lever pressing.

EVALUATION

The effect of a drug on the performance of an animal is compared to the data generated in the previous non-drug sessions. Each animal thereby serves as its own control. The basic measures of performance during a specific time interval, responses and shocks are used for evaluation. Responses are reported both as total and as percent of control responses. Shocks are reported as totals and as shock-avoided (SHA) as percent of control. This latter measure is computed by subtracting the number of shocks received from the total number of possible shocks if no responses had been made. In the initial screening of experimental compounds, the results are reported in terms of the total effect during a five-hour test. However, an ED_{50} is usually estimated during a representative time of peak activity.

CRITICAL ASSESSMENT OF THE METHOD

Anxiolytics show activity in this test, however, it has been proven to be more reliable for neuroleptic activity. Apparently, the present conditioned active avoidance paradigms do not constitute a reliable method for screening anxiolytic agents, in spite of their homologies with human anxiety.

MODIFICATIONS OF THE METHOD

Heise and Boff (1962) and Galizio et al. (1990) extended the method by using two levers, an "avoidance lever" and an "escape lever" for calculating ratios between shock rate, escape failure rate and avoidance rate.

Balfour (1990) described the effect of drugs on rat behavior in an unsignalled Sidman avoidance schedule.

Wadenberg et al. (1998) described and evaluated a newly designed apparatus for the assessment of conditioned avoidance response performance in rats.

Patel and Migler (1982) reported a sensitive and selective conflict test in **squirrel monkeys**.

Szewczak et al. (1995) tested antipsychotic agents using continuous avoidance behavior in adult male squirrel monkeys.

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E.2.5.2 Geller conflict paradigm

PURPOSE AND RATIONALE

Experimentally induced conflict by punishing food-rewarded behavior has been used to differentiate between various psychoactive drugs by Geller and Seifter (1960). The basic principle has been used and modified by many authors to reveal possible anti-anxiety effects of experimental compounds.

PROCEDURE

Male albino rats with a starting body weight of 300–400 g are housed individually. They are food deprived until the body weight is gradually reduced by approximately 20% of original and it is maintained at this level by restricted food diet. Conditioning is carried out in commercially available Skinner boxes (e.g. Campden Instruments, London, UK) equipped with a house light, a single lever, cue lights, a liquid dripper, and a grid-floor connected to a shocker. Sweetened condensed milk delivered by the liquid dipper serves as the positive reinforcer. The data are recorded on cumulative recorders.

The animals are trained to lever press for the milk reward in two distinct response-reward sections. In the anxiety or "conflict" segment (signalled by onset of both tone and cue lights), a dipper of milk is delivered in response to each lever press (continuos reinforcement schedule = CRF) However, lever presses during this period are also accompanied by a 40-ms pulse of aversive foot-shock through the grid floor. This creates a conflict between milk reward and the a painful foot shock. This conflict period is 3 min in duration.

During the other segment of this paradigm, the lever presses produce a drop of milk only at variable intervals of time from 60 to 210 s with an average reward of once per 2 min (variable interval = VI-2 min). No shocks are administered during this variable interval phase of testing which is 15 min in duration.

The test procedure consists of four 15 min nonshock variable interval segments where reinforcement is available on a restricted basis. Each variable interval period is followed by a three minute CRF-conflict period phase when reinforcement is constantly available but always accompanied by an aversive footshock. The shock level is adjusted for each subject to reduce the CRF responding to a total of less than 10 lever presses during the entire test. The rats are tested 2 to 4 days a week. Drugs are administered once per week and the performance is compared to the previous day's control trial. The VI responses are used to evaluate any general debilitating drug effects while the CRF responses are used to evaluate any anti-anxiety effects as indicated by the increased responding during the CRF conflict period.

The test compounds are administered intraperitoneally 30 min or orally 60 min before the test period.

EVALUATION

The total number of lever presses during the conflict periods (CRF) and the non-conflict periods (VI) are counted. Values of treatment sessions are expressed as percentage of values of the preceding non-treatment day. An increase of lever presses in the conflict period is regarded as indication of an anti-anxiety effect, and a decrease of lever presses in the non-conflict period as an indication for a sedative effect. In this procedure linear dose-response curves are rarely found. Therefore, minimal effective doses (MED) are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method is suitable to distinguish anxiolytics from other centrally active drugs, such as sedatives and neuroleptics. The relative potency of various anxiolytic agents in a number of species compares favorably with their relative potency in humans. The negative aspects are the time consuming procedure, the high expenses for the apparatus and the difficulty to obtain ED_{50}-values.

The relevance of this test and other models has been challenged by Bignami (1988).

MODIFICATIONS OF THE METHOD

The method has been modified by many authors.

Davidson and Cook (1969), Cook and Davidson (1973) introduced a schedule of 10 lever presses for the delivery of one food pellet together with an electric foot-shock. In the variable interval reinforcement period, responses were reinforced by food delivery at varying intervals of time; the mean interval was 30 s. Also under these conditions differentiation of various psychotropic drugs could be achieved.

Iorio et al. (1986) and Chipkin et al. (1988) used a procedure during which a tone (5 s) preceded and then overlapped for 10 s with scrambled footshocks. After the start of the tone, the rat has the option to avoid (in the no shock period) or escape (in the shock period) the shock by jumping onto a platform located 17 cm above the grid floor.

Thiébot et al. (1991) developed the method further by introducing "safety signal withdrawal", a behavioral paradigm in rats sensitive to both anxiolytic and anxiogenic drugs.

Commissaris and Fontana (1991) published a potential animal model for the study of antipanic treatments. Rats were trained to drink their daily water ration during 10-minute sessions. These sessions were characterized by alterations of silence (unpunished periods) and the presence of a tone (punished periods). Tube contact during the tone periods resulted in a 0.25–0.5 mA shock delivered to the mouth of the animal for the duration of the tube contact. After 4 weeks of training, subjects received either chronic post-test treatment with an antidepressant or vehicle, twice daily, seven days per week. Acute pre-test administration of traditional anxiolytics (benzodiazepines, barbiturates) increased dramatically the number of punished contacts made in a dose-dependent manner. Typical antidepressants were not active after pre-test administration, however, active after chronic post-test treatment.

The conflict test has also be adapted to the **mouse** (Prado de Carvalho et al. 1986).

The conflict behavior and anticonflict effect of anxiolytics has been demonstrated in a variety of species, including **pigeons** (Morse 1964; Wuttke and Kelleher 1970; McMillan 1973; Gleeson et al. 1989; Barrett et al. 1989; Barrett 1991; Schipper et al. 1991; Pollard et al. 1992; Barrett et al. 1994; Mos et al. 1997).

Patel and Migler (1982) described a sensitive and selective conflict model in male squirrel **monkeys** in which anti-anxiety agents exhibit pronounced anticonflict activity.

Ervin et al. (1987), Ervin and Cooper (1988), van Heest et al. 1991; Simiand et al. (1993) used **conditioned taste aversion** as a conflict model. Moderate taste aversions were induced by pairing the initial consumption of 0.25% sodium saccharin with either 25 mg/kg 5-hydroxytryptophan or 30 mg/kg i.p. LiCl. Antagonism was found with benzodiazepines and non-benzodiazepine-anxiolytic drugs.

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E.2.5.3 Conditioned defensive burying in rats

PURPOSE AND RATIONALE

Besides the well known defensive reactions of animals in typical laboratory settings, like freezing, fleeing, and attacking, rats tested in the presence of bedding material develop a peculiar behavior when shocked through a stationary prod by burying the shock source. This behavior has been proposed as a new paradigm for the study of anxiolytic agents (Pinel and Treit 1978, 1983; Treit et al. 1981).

PROCEDURE

Male adult rats weighing 250–400 g are used. The testing is performed in $44 \times 30 \times 44$ cm Plexiglas test chambers, the floor of which is covered with 5 cm of a commercial bedding material. In the center of each of the four walls, 2 cm above the level of the bedding material, is a small hole though which a $6.5 \times 0.5 \times 0.5$ cm wire-wrapped wooden dowel (i.e., the shock prod) can be inserted. Electric current is administered through the two uninsulated wires wrapped around the prod. The behavior of each rat is monitored for 15 min from a separate room via closed circuit television.

Before each of the experiments, the rats are placed in the Plexiglas test chamber in groups of 5 or 6 for 30-min periods on each of 4 consecutive days. In the experiments, groups of 10 animals are used for each dose and control. The rat is injected intraperitoneally with the test drug or saline before being placed into the test chamber in which the shock prod is inserted. The animal is placed into the center of the chamber so that is faces away from the prod. When the rat first touches the prod with its forepaw, it receives a brief electric shock (1 mA) which typically elicits a flinch away from the prod and withdrawal towards the back of the chamber. Afterwards, the rat moves directly towards the prod, pushing and spraying a pile of bedding material ahead with rapid shoveling movements of its snout and alternating pushing movements of its forepaws. The prod is buried in a pile of bedding material. The duration of burying is recorded.

EVALUATION

The mean duration of burying in treated animals is compared with controls. Anxiolytics as well as neuroleptics shorten the burying behavior dose-dependent at low shock intensity (1 mA). A second measure is the height of the bedding material. At high shock intensity (10 mA) only neuroleptics but not benzodiazepines are active.

CRITICAL ASSESSMENT OF THE METHOD

The method of conditioned defensive behavior can be regarded as a simple and reliable method to detect anxiolytic activity of benzodiazepines and neuroleptics. However, using higher shock intensity – where only chlorpromazine was found to be active – a separation between benzodiazepines and neuroleptics has been demonstrated.

The drug-class specificity of the test has been challenged by Craft et al. (1988).

MODIFICATIONS OF THE METHOD

Diamant et al. (1991) used telemetry to register autonomic and behavioral responses in the shock-prod burying test in rats.

Wiersma et al. (1996) reported that microinfusion of corticotropin-releasing hormone in the central amygdala of freely moving rats enhanced the active behavior responses in the conditioned defensive burying paradigm.

Fernandez-Guasti and Lopez-Rubalcava (1998) used the rat burying behavior test to study the effect of various potential anxiolytics.

Broekkamp et al. (1986), Njung'e and Handley (1991a,b; Gacsályi et al. 1997) described burying of marbles by *mice* as harmless objects without punishment by electrical shocks as a model for detection of anxiolytics. Thirty min after treatment with drugs, male Swiss mice, weighing 20–24 g, were individually placed in a $23 \times 17 \times 14$ cm cage with 25 glass marbles, 1.5 cm in diameter. The glass marbles were placed in close contact in the middle of the cage on a 5 cm layer of sawdust. The mice were left in the cage with the marbles for 30 min after which the test was terminated by removing the mice and counting the number of marbles that were more than two thirds covered with sawdust.

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E.2.5.4 Taste aversion paradigm

PURPOSE AND RATIONALE

When ingestion of a taste stimulus is paired with internal malaise the animal remembers the taste and rejects its ingestion thereafter. This phenomenon is called a conditioned taste aversion or taste aversion learning. In the classical experiment in rats, the conditioned stimulus is a 0.01 M drinking solution of saccharin paired with an intraperitoneal injection of 0.15 M LiCl solution as unconditioned stimulus. The underlying neural mechanisms (Yamamoto 1993; Agüero et al. 1993, 1996; Swank et al. 1995) as well as facilitating and

inhibiting factors (Lipinski et al. 1995; Sobel et al. 1995) were investigated. Moreover, drugs by themselves can be studied. Rats are presented a fluid with palatable taste and immediately after consumption of this liquid injected with a drug, whose effect the animals have not experienced before. Subsequently, on a later occasion and under non-drug conditions, avoidance of the taste associated with the drug is measured (De Beun et al. 1996).

PROCEDURE

Male Wistar rats weighing 220–250 g are housed in groups of four per cage under a normal 12L:12D regime at 22–23 °C with free access to food and water. Twenty-four hours before the first conditioned taste aversion session, the animals are water deprived and fluid access is from then on restricted to daily experimental sessions of 15 min which takes place individually in a test cage. After each session, the animals are returned to their home cages. Food is freely available in the home cages throughout the procedure, but not available during the sessions. For a given subject, all six session required to complete a conditioned taste aversion experiment take place in the same test cage. Animals designated to the same experimental group are run in parallel. During the first four sessions (day 1 through day 4), both bottles contain plain tap water. During this phase of the procedure, the animal learns to drink a reasonable amount of fluid in a short period of time. For the 5th session (day 5, conditioning session), both bottles are filled with a 0.1% saccharin solution and immediately after completion of this session the animals are injected with either the vehicle or different doses of the test drug. Per animal, only one dose (or the vehicle) of a particular drug is tested. On days 6 and 7, no sessions are conducted (washout period) and the animals have free access to tap water in the home cages from the end of day 5 until the morning of day 7, when the animals are again deprived of water, 24 h prior to the final 6th session (day 8, test session). During this last session, one bottle contains the saccharin solution and the other bottle is filled with tap water. To control for location bias, the saccharin is presented in the left bottle for half of the animals in each group and in the right bottle for the other half. By measuring the amount of fluid consumed from both bottles separately, drug-induced conditioned taste aversion can be determined by comparison of the relative saccharin intake in the drug treated groups and their vehicle-treated controls.

EVALUATION

Data of test drugs are submitted separately to one-way analysis of variance, with the between-subjects factor DOSE. The dependent variable is the ratio of (saccharin solution/saccharin solution + tap water) intake. Fluid intake scores are calculated in grams. Post hoc analyses are used with Tukey HSD multiple comparisons. Results are considered significant when $p < 0.05$.

MODIFICATIONS OF THE METHOD

Besides LiCl, several other drugs were used to induce taste aversion, such as ethanol (Gauvin and Holloway 1992; June et al. 1992; Thiele et al. 1996; Bienkwoski et al. 1997), morphine (Miller et al. 1990; Bardo and Valone 1994), cocaine (Van Haaren and Highes 1990; Glowa et al. 1994), naloxone (Mucha 1997), apomorphine (McAllister and Pratt (1998), caffeine (Brockwell et al. 1991), d-amphetamine (Davies and Wellman 1990; Lin et al. 1994), nicotine (Shoaib and Stolermman 1996), quinine (Parker 1994), cisplatin (Mele et al. (1992), benzodiazepine and non-benzodiazepine anxiolytics (Neisewander et al. 1990), 5-hydroxytryptamine (Rudd et al. 1998), dopamine D_3 agonists (Bevins et al. 1996), cyclosporine A (Exton et al. 1998), cholecystokinin (Ervin et al. 1995; Mosher et al. 1996) and Δ9-tetrahydrocannabinol (Parker and Gillies 1995).

Turenne et al. (1996) found individual differences in reactivity to the aversive properties of drugs. Rats were assigned to high conditioned taste avoidance and low conditioned taste avoidance groups on the basis of their intake of saccharin solution previously paired with morphine, amphetamine, lithium, or fenfluramine.

Willner et al. (1992) investigated the influence of drugs on taste-potentiated odor aversion learning in rats.

Rabin and Hunt (1992) studied taste aversion learning in **ferrets**.

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E.2.6
Effects on the endocrine system

E.2.6.1
Plasma catecholamine levels during and after stress

PURPOSE AND RATIONALE

A wide variety of stressors causes significant biochemical, physiological, and behavioral changes. These changes include marked increases in plasma catecholamines, heart rate and blood pressure. Treatment with anxiolytic drugs can attenuate the stress induced increases of norepinephrine and epinephrine in plasma (Vogel et al. 1984; Livesey et al. 1985; Taylor et al. 1989; de Boer et al. 1990; Krieman et al. 1992).

PROCEDURE

Male Sprague-Dawley rats, 300–350 g, are individually housed and given food and water ad libitum. After 1 week of acclimatization, an aortic catheter is surgically implanted in each animal, running on top of the psoas muscle and brought out through an incision at the back of the neck. The catheters are flushed

daily with heparinized saline. After a recovery period of 48 h baseline (time –15) blood samples are drawn and blood pressure recordings are taken using a Grass model 7 polygraph. The animals are then given an i.p injection of the test compound or the vehicle. 15 min after the injection (time 0), blood samples, and heart rate and blood pressure measurements are taken. Animals in control and treatment groups are then stressed by immobilization for 1 h and blood samples, heart rate and blood pressure recordings are taken at time 15, 30 and 60 min during stress. Immobilization is performed by taping the legs of the animals to the laboratory bench. After the stress period of 1 h, the animals are released, returned to the home cage for recovery and 1 h post-stress samples and recordings are taken. Non-stressed, compound or vehicle treated animals remain in the home cage for the entire test period and are sampled in the same manner as the stressed animals. Approximately 0.3 ml blood is withdrawn for each sample and an equal amount of 0.9% saline is re-infused to prevent changes in blood volume.

EVALUATION

Determinations of norepinephrine and epinephrine in plasma are made using an radio-enzymatic assay. Heart rate and systolic and diastolic blood pressure are determined directly from the polygraph tracings. The data are analyzed using a three-way analysis of variance (ANOVA) with repeated measures, two-way ANOVA, Students *t*-test, Student-Newman-Keuls and the trapezoidal rule for the area under the curve.

CRITICAL ASSESSMENT OF THE METHOD

Reduction of catecholamine levels but no changes of the cardiovascular parameters could be found after treatment with anxiolytics. The test can be used as a method for evaluation of the influence of psychoactive drugs on the endocrine system (Krieman et al. 1992).

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E.2.6.2 Plasma corticosterone levels influenced by psychotropic drugs

PURPOSE AND RATIONALE

Corticosterone levels in the blood of rats are elevated not only after stress but also after application of selective 5-HT receptor agonists (Koenig et al. 1987). This has been used to differentiate typical and atypical neuroleptics (Nash et al. 1988).

PROCEDURE

Male Sprague Dawley rats weighing 200–250 g are housed 6 per cage under temperature and light/dark controlled conditions with free access to food and water. On the day prior to an experiment, the animals are transferred to the experimental room. On the day of the experiment, various doses of test drugs or saline are injected intraperitoneally. After 60 min 2.5 mg/kg 6-chloro-2-(1-piperazinyl)pyrazine (MK-212) are injected intraperitoneally followed by decapitation after further 60 min. Trunk blood is collected and allowed to clot. Serum is obtained following centrifugation and stored at –20 °C for the radioimmunoassay of corticosterone.

EVALUATION

Data are analyzed with a two-way analysis of variance. Differences between treatment groups are evaluated using the Student-Newman-Keuls test.

MODIFICATIONS OF THE METHOD

Korte et al. (1991) studied the effect of a 5-HT_{1A} agonist on behavior and plasma corticosterone levels in male Wistar rats before and after psychological stress of defeat.

Broqua et al. (1992) measured corticosterone and glucose levels in blood together with parameters of 5-HT metabolism in brain in stressed animals treated with the anti-depressant tianeptine.

Rittenhouse et al. (1992) measured plasma concentrations of renin, corticosterone, ACTH, and prolactin in rats after treatment with a 5-HT_{1A} agonist in three

stress paradigms: immobilization, forced swim and conditioned fear.

Groenink et al. (1995) studied the corticosterone secretion in rats after application of 5-HT_{1A} receptor agonists and antagonists.

Aulakh et al. (1988, 1993) reported higher baseline levels of plasma corticosterone in fawn-hooded rats relative to Wistar and Sprague-Dawley strain rats. Long-term treatment (21 days) with antidepressant drugs significantly decreased plasma corticosterone in fawn-hooded rats. The authors recommended this strain as a genetic model of depression.

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E.2.7 Benzodiazepine dependence

E.2.7.1 General considerations

Benzodiazepine dependence is a hypothetical construct for the adaptive changes that occur as a result of chronic drug exposure. Two measures are usually considered to reflect dependence: the development to a drug's effects, and the abstinence signs of drug withdrawal.

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E.2.7.2 Benzodiazepine tolerance and dependence in rats

PURPOSE AND RATIONALE

Induction of benzodiazepine tolerance and physical dependence has been reported for several animal species, such as rats, mice, dogs and monkeys. Ryan and Boisse (1983) and Boisse et al. (1986) developed a reproducible model exemplified for chlordiazepoxide in rats.

PROCEDURE

Male Sprague Dawley rats weighing 350–575 g are used. Chordiazepoxide hydrochloride is administered as solution 75 mg/ml by gavage after an initial loading dose of 450 mg/kg given at 7:00 A.M. and 5:00 P.M. Impairment of motor function is evaluated by a neurological screen including five different ladder and open field tests.

Neurological test	Maximum depression points
Ladder, head down	2
Ladder, head up	4
Ladder, grasp reflex	1
Motor activity	3
Walking	5
Maximum	15

The animals are rated by three independent observers. Once dependence is revealed, lower doses are used which are then increased in appropriate steps to induce an average depression rating of about five. Treatment is continued for 5 weeks during which time the dose has to be increased. The degree of increase (about 5-fold for in the case of chlordiazepoxide) reflects the **tolerance** of the test compound.

To test **dependence**, rats are challenged with the benzodiazepine receptor antagonist Ro 15-1 788 (flumazenil; ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-

imidazo(1,5a)(1,4)benzodiazepine-3-carboxylate) at a dose of 25 mg/kg i.p. Withdrawal reactions are recorded just before, and 5, 15, 30 and 60 min after administration of the antagonist. Motor, autonomic and behavioral signs are monitored by operational defined criteria, such as position of the claws, salivation and diarrhea. The total withdrawal expression for each observation and each animal is estimated by summing the grades of all signs as recorded by three independent observers.

EVALUATION

For each animal and observation time, the scores are estimated by the average of all co-observers. From these estimates group means are computed and compared by *t*-test between control and test and by paired *t*-test for self-control comparisons before and after antagonist administration

MODIFICATIONS OF THE METHOD

Further studies in *rat* with benzodiazepines were preformed by Vellucci and File (1979), Treit (1985) and Nath et al. (1997).

File (1985) found very different rates at which tolerance develops to the sedative, anticonvulsive and anxiolytic actions of benzodiazepines.

Bonnafous et al. (1995) studied the increase of gastric emptying induced by benzodiazepine withdrawal in rats. Gastric emptying was measured with a test meal containing ^{51}Cr sodium chromate administered in rats, either previously receiving 15 mg/kg diazepam or DMSO i.p. for 7 days.

Benzodiazepine-like dependence potential of a putative 5-HT_{1A} agonist anxiolytic was assessed in rats by Goudie et al. (1994).

Studies in *mice* on tolerance and physical dependence with benzodiazepines were performed by Patel et al. (1988), Gallaher et al. (1986), Stephens and Schneider (1985), Nutt and Costello (1988) and Piot et al. (1990).

These phenomena were studied in *dogs* by McNicolas et al. (1988) and Löscher et al. (1989).

Studies in monkeys were performed by Lukas and Griffiths (1982), Yanagita (1983), Lamb and Griffiths 1984) and France and Gerak (1997).

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E.3 Anti-epileptic activity

E.3.0.1 General considerations

Epilepsy is a disease of high prevalence, being well known since thousands of years as “morbus sacer”. In spite of intensive investigations, the pathophysiology of epilepsy is still poorly understood. Studies with various animals models have provided ample evidence for heterogeneity in the mechanisms of epileptogenesis.

New evidence derives from investigations of kindling, which involves the delivery of brief, initially subliminal, electrical or chemical stimuli to various areas of the brain. After 10 to 15 days of once-daily stimulation, the duration and intensity of after-discharges reach a stable maximum and a characteristic seizure is produced. Subsequent stimulation then regularly elicits seizures.

Surveys of methods being used to test compounds with anticonvulsant properties have been provided by Toman and Everett (1964), Woodbury (1972), Hout et al. (1973), Swinyard (1973), Koella (1985), Meldrum (1986), Rump and Kowalczyk 1987; Löscher and Schmidt (1988), Fisher (1989), Rogawski and Porter (1990), Porter and Rogawski (1992).

Several biochemical hypotheses have been advanced, involving the inhibitory GABAergic system and the system of the excitatory amino acids glutamate and aspartate. Excitatory receptors have been divided into subtypes according to the actions of specific agonists or antagonists. Agents which reduce $GABA_A$ synaptic function provoke convulsions. A convulsive state is induced by the direct blockade of $GABA_A$ receptors (e.g. to the action of bicuculline) or a reduction in the GABA-mediated opening of the chloride ion channel (e.g., by picrotoxin). One major factor in epileptogenesis seems to be a decreased function of $GABA_A$ synapses.

More recently, research has focused on the therapeutic potential of blocking excitatory amino acids – in particular glutamate. Of the three receptors of glutamate, the NMDA (N-methyl-D-aspartate)-receptor is considered the one of most interest in epilepsy and competitive NMDA receptor antagonists are proposed as potential anti-epileptic drugs. Excessive excitatory amino acid neurotransmission is thought to be associated with the neuropathologies of epilepsy, stroke and other neurodegenerative disorders. Antagonism of NMDA receptor function appears to be the mechanism of action of some novel anticonvulsant and neuroprotective agents. Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA) and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987).

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E.3.1 *In vitro* methods

E.3.1.1 ^{3}H-GABA receptor binding

See Sect. E.2.1.1.

E.3.1.2 $GABA_A$ receptor binding

See Sect. E.2.1.2.

E.3.1.3 $GABA_B$ receptor binding

See Sect. E.2.1.3.

The *in vitro* assays for GABA-ergic compounds, described in the Sect. E.2 (anxiolytics) are similarly used for evaluation of anti-epileptic compounds.

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E.3.1.4 ^{3}H-GABA uptake in rat cerebral cortex synaptosomes

PURPOSE AND RATIONALE

Roberts (1974) and others have proposed that the inhibitory action of the amino acid γ-aminobutyric acid (GABA) is the fine tuning control for pacemaker neurons. Disruption of this interplay due to inadequacies of the GABA system result in various disorders, in particular convulsive seizures (Roberts 1974; Korgsgaard-Larsen 1985). The nonspecific action of GABA-mimetics makes inhibition of the uptake mechanism, which terminates the neurotransmitters action, the ideal choice for increasing GABA's concentration at specific sites (Roberts 1974; Tapia 1975; Meldrum et al. 1982; Brehm et al. 1979). Demonstration of the high affinity mechanism that best reflects the *in vivo* condition utilizes GABA depleted cerebral cortex synaptosomes (Ryan and Roskoski 1977; Iversen and Bloom 1972; Roskoski 1978). Although the physiological role of GABA transport systems is still unclear, uptake inhibitors such as THPO [4,5,6,7-tetrahydroisoxazolo- (4,5-C)pyrid-3-ol], nipecotic acid, cis-4-hydroxynipe-cotic acid, and guvacine exhibit anticonvulsant effects (Meldrum et al. 1982; Brehm et al. 1979). Furthermore, a number of neuroleptics have been shown to inhibit GABA uptake (Fjalland 1978). In particular, fluspirilene was found to be equivalent to the most potent uptake inhibitors known.

The assay is used as a biochemical screen for potential anticonvulsants or GABA (γ-aminobutyric acid) mimetic compounds that act by inhibiting GABA uptake.

PROCEDURE

Reagents

1. 0.5 M Tris buffer, pH 7.4
2. Ringer's solution + 10 mM Tris buffer, pH 7.4 containing
   - glucose 10.0 mM,
   - NaCl 150.0 mM,
   - KCl 1.0 mM,
   - $MgSO_4$ 1.2 mM,
   - Na_2HPO_4 1.2 mM.
3. Depolarizing Ringer's solution, pH 7.4 reagent 2 containing
   - KCl 56 mM,
   - $CaCl_2$ 1 mM.
4. 0.32 M sucrose
5. ^{3}H-GABA is diluted to 2.5×10^{-4} M with distilled water. Forty µl of this solution in 1 ml of reaction mixture will yield a final concentration of 10^{-5} M.
6. Test compounds
   A 10 mM stock solution is made up in distilled water, ethanol, or DMSO and serially diluted, such that the final concentration in the assay ranges from 10^{-3} to 10^{-8} M. Total and nonspecific controls should use solvent of test compound.

Tissue preparation

Male Wistar rats are decapitated and the brains rapidly removed. Cerebral cortex is weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose using a Potter-Elvejhem homogenizer. The homogenate is centrifuged at 1000 *g* for 10 min. The supernatant (S_1) is decanted and recentrifuged at 1000 *g* for 10 min. The pellet (P_2) is resuspended in 9 volumes of 0.32 M sucrose and centrifuged at 24000 *g* for 10 min. The washed pellet is resuspended in15 volumes of depolarizing Ringer's solution, incubated at 25 °C for 10 min and centrifuged at 3000 *g* for 10 min. The resulting pellet is resuspended in 15 volumes of Ringer's solution and is ready for use.

Assay

60 µl Ringer's solution,
100 µl vehicle or appropriate drug concentration,
800 µl tissue suspension.

Microcentrifuge tubes are set up in triplicate. Nonspecific controls are incubated at 0°C and totals at 25 °C for 10 min. Forty µl of ^{3}H-GABA are added and the tubes are reincubated for 10 min. All tubes are centrifuged at 13000 *g* for 1 min. The supernatant is aspirated and 1 ml of solubilizer (Triton X-100 + 50% EtOH, 1:4, v/v) is added and mixed to dissolve pellets. Tubes are incubated at 90 °C for 3 min, then centrifuged at 13000 *g* for 15 min. 40 µl of supernatant is counted in 10 ml Liquiscint scintillation cocktail.

EVALUATION

Active uptake is the difference between cpm at 25 °C and 0 °C. The percent inhibition at each drug concentration is the mean of three determinations. IC_{50} values are derived from log- probit analysis.

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E.3.1.5 GABA uptake and release in rat hippocampal slices

PURPOSE AND RATIONALE

The GABA transporter, the subsynaptic $GABA_A$-receptor, and the $GABA_B$-autoreceptor are therapeutically the most relevant targets for drug actions influencing GABAergic synaptic transmission. Uptake inhibitors are potential anticonvulsants.

PROCEDURE

For measurement of GABA uptake, rat hippocampal slices are cut with a McIlwain tissue slicer (100 µm-thick prisms) and dispersed in ice-cold Krebs-Ringer solution with HEPES buffer (pH 7.4). Following two washes, slices (15 mg) are incubated at 37 °C for 15 min in the presence or absence of test compound. [^{3}H]-GABA is added and samples are incubated for an additional 5 min before filtration through Whatman GF/F filters. Samples are then washed twice with 5 ml ice-chilled 0.9% saline. Distilled water is added and samples are allowed to sit at least 60 min before measured for radioactivity by liquid scintillation spectroscopy. Blanks are treated in an identical manner but are left on ice throughout the incubation.

For measurement of GABA release, rat hippocampal slices are prepared and dispersed in ice-cold HEPES-buffered (pH 7.2) Krebs Ringer solution and incubated with 0.05 µM [^{3}H]-GABA for 15 min at 37 °C. Following two washes, the slices are incubated for an additional 15 min and finally resuspended in medium. Tissue (10 mg) is incubated at 37 °C for a 15 min release period in the presence or absence of test compound. At the end of the release period, the medium is separated from tissue by centrifugation at 500 *g* for approximately 1 min and poured into 0.5 ml of perchloric acid (0.4 N). The tissue is homogenized in 0.13 N perchloric acid. Radioactivity in the samples is measured by using liquid scintillation spectroscopy.

EVALUATION

For GABA-uptake, IC_{50}-values (µM) are determined.

In GABA-release experiments, results are expressed as the amount of radioactivity released as a percent of the total radioactivity.

MODIFICATIONS OF THE METHOD

Roskoski (1978) studied the net uptake of GABA by high affinity synaptosomal transport systems.

Nilsson et al. (1990, 1992) tested GABA uptake in astroglial primary cultures.

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E.3.1.6 Glutamate receptors: [^{3}H]CPP binding

PURPOSE AND RATIONALE

This assay is used to assess the affinity of compounds for the excitatory amino acid binding site of the NMDA (N-methyl-D-aspartate) receptor complex. [^{3}H]CPP (3-[(±)-2-carboxypiperazin-4-yl]-1-phosphonic acid) is a structurally rigid analog of the selective NMDA receptor antagonist 2-AP7 (2-amino-7-phosphonoheptanoic acid).

Excessive excitatory amino acid neurotransmission has been associated with the neuropathologies of epilepsy, stroke and other neurodegenerative disorders (Cotman and Iversen 1987; Watkins and Olverman 1987; Parsons et al. 1998). Antagonism of NMDA receptor function appears to be the mechanism of action of some anticonvulsant and neuroprotective agents (Loscher 1998; Tauboll and Gjerstad 1998). Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA) and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987; Collingridge and Lester 1989; Monaghan et al. 1989; Carlsson and Carlsson 1990; Young and Fagg 1990; Nakanishi 1992; Cunningham et al. 1994; Herrling 1994; Iversen and Kemp 1994; Mayer et al. 1994; Meldrum and Chapman 1994; Monaghan and Buller 1994; Watkins 1994; Bettler and Mulle 1995; Fletcher and Lodge 1995; Becker et al. 1998; Danysz and Parsons 1998; Meldrum 1998; Chittajallu 1999; Dingledine et al. 1999; Hatt 1999; Gallo and Ghiani 2000; Lees 2000; Meldrum 2000). The binding site for [^{3}H]2-amino-4-phosphonobutyric acid (AP4) may represent a fourth site which is less well characterized (Thomsen 1997). NMDA receptors are believed to be coupled to a cation channel which converts to an open state with NMDA receptor activation (Kemp et al. 1987; Mukhin et al. 1997). The opening and closing of this cation channel are also modulated by glycine, Mg^{2+} and Zn^{2+}. Dissociative anesthetics, such as phencyclidine (PCP) and ketamine, and novel anticonvulsants, such as MK-801, block the ion channel and are noncompetitive NMDA receptor antagonists. Competitive NMDA receptor antagonists, such as CPP and the phosphono analogs of L-glutamate, AP7 and AP5 (2-amino-5-phosphonopentanoic acid), are inhibitors at the excitatory amino acid binding site (Olverman et al. 1986; Davies et al. 1986; Harris et al. 1986; Murphy et al. 1987; Lehmann et al. 1987).

PROCEDURE

Reagents

1. Buffer A: 0.5 M Tris HCl, pH 7.6
   60.0 g Tris HCl
   13.9 g Tris Base
   q.s. to 1 liter with distilled water
2. Buffer B: 50 mM Tris HCl, pH 7.6
   Dilute buffer A 1 : 10 with distilled water
3. L-Glutamic acid, 5×10^{-3} M
   Dissolve 7.36 mg of l-glutamic acid (Sigma G1 251) with 10.0 ml distilled water. Aliquots of 20 µl to the assay tube will give a final concentration of 10^4M.
4. [^{3}H]CPP is obtained from New England Nuclear, specific activity 25–30 Ci/mmol. For IC_{50} determinations, a 200 nM stock solution is made with distilled water. Aliquots of 50 µl are added to each tube to yield a final concentration of 10 mM.
5. Test compounds. A stock solution of mM is made with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.
6. Triton-X 100, 10% (v/v) (National Diagnostics, EC-606) A stock solution of Triton-X100, 10%, can be prepared and stored in the refrigerator. Dilute 1.0 ml of Triton-X100 to 10.0 ml with distilled water. On the day of the assay, the tissue homogenate (1 : 15 dilution) is preincubated with an aliquot of Triton-X 100, 10%, to give a final concentration of 0.05% (v/v).

Tissue preparation

Cortices of male Wistar rats are dissected over ice and homogenized in ice-cold 0.32 M sucrose, 15 volumes of original wet weight of tissue, for 30 s with a Tissumizer setting at 70. The homogenate is centrifuged at 1 000 *g* for 10 min. (SS34, 3 000 rpm, 4 °C). The supernatant is centrifuged at 20 000 *g* (SS34, 12 000 rpm, 4 °C) for 20 min. Resuspend the pellet in 15 volumes of ice-cold distilled water (Tissumizer setting 60, 15 s) and spin at 7 600 *g* (SS34, 8 000 rpm, 4 °C) for 20 min. Save the supernatant, swirl off the upper buffy layer of the pellet and add to the supernatant. Centrifuge the supernatant at 48 000 *g* (SS34, 20 000 rpm, 4 °C) for 20 min. Resuspend the pellet with 15 volumes of cold distilled water and centrifuge. Discard the supernatant and store the pellet at –70 °C.

On the day of the assay, resuspend the pellet in 15 volumes ice-cold 50 mM Tris buffer, pH 7.6. Preincubate the homogenate with Triton-X in a final concentration

0.05% (v/v) for 15 min at 37 °C with agitation. Centrifuge the homogenate at 48 000 *g* (SS34, 20 000 rpm, 4 °C for 20 min. Wash the pellet an additional 3 times by resuspension with cold buffer and centrifugation. The final pellet is resuspended in a volume 20 times the original wet weight.

Assay

1. Prepare assay tubes in triplicate.
   - 380 µl distilled water
   - 50 µl buffer A, 0.5 M Tris HCl, pH 7.6
   - 20 µl 1-glutamic acid, 10^{-4} M, or distilled water or appropriate concentration of inhibitor
   - 50 µl [^{3}H]CPP
   - 500 µl tissue homogenate
2. Following the addition of the tissue, the tubes are incubated for 20 min at 25 °C with agitation. Place the tubes in an ice bath at the end of the incubation. Terminate the binding by centrifugation (HS4, 7 000 rpm, 4 °C) for 15 min. Return the tubes to ice. Aspirate the discard the supernatant. Carefully rinse the pellet three times with 1 ml ice-cold buffer, avoiding disruption of the pellet. Transfer the pellet to scintillation vials by vortexing the pellet with 2 ml scintillation fluid, rinse the tubes twice with 2 ml and add an additional 4 ml scintillation fluid.

EVALUATION

Specific binding is determined from the difference of binding in the absence of presence of 10^{-4} M L-glutamic acid and is typically 60–70% of total binding. IC_{50} values for the competing drug are calculated by log-probit analysis of the data.

MODIFICATIONS OF THE ASSAY

Glutamate (non selective)

The assay measures the binding of glutamate, which binds non selectively to ionotropic glutamate receptors including the NMDA, AMPA, and kainate subtypes (Foster and Fagg 1987). In addition, glutamate binds to a family of metabotropic glutamate receptors.

Whole brains (except cerebellum) are obtained from male Wistar rats. A membrane fraction is prepared by standard techniques. Ten mg of membrane preparation is incubated with 1.6 nM [^{3}H]L-glutamate for 10 min at 37 °C. Non-specific binding is estimated in the presence of 50 µM L-glutamate. Membranes are filtered and washed 3 times to separate bound from free ligand and filters are counted to determine [^{3}H]L-glutamate bound.

Convulsions induced in mice by intravenous injections of 2.0 mmol/kg L-glutamic acid can be inhibited by glutamate antagonists (Piotrovsky et al. 1991).

Glutamate AMPA

The assay measures the binding of [^{3}H]AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), a selective agonist which binds to the AMPA receptor subtype of glutamate-gated ion channels (Honore et al. 1982; Olsen et al. 1987; Fletcher and Lodge 1995).

Membranes are prepared from male rat brain cortices by standard techniques. Fifteen mg of membrane preparation is incubated with 5 nM [^{3}H]AMPA for 90 min at 4 °C. Non-specific binding is estimated in the presence of 1 mM L-glutamate. Membranes are filtered and washed 3 times and the filters are counted to determine [^{3}H]AMP bound.

Mutel et al. (1998) recommended [^{3}H]Ro 48-8 587 as specific for the AMPA receptor.

Fleck et al. (1996) described AMPA receptor heterogeneity in rat hippocampal neurons. AMPA receptor antagonists were described by Kohara et al. (1998), Wahl et al. (1998), Kodama et al. (1999), Nielsen et al. (1999) and reviewed by Chimirri et al. (1999).

Glutamate Kainate

The assay measures the binding of [^{3}H]kainate, a selective agonist that binds to the kainate subtype of the ionotropic glutamate receptors in rat brain (London and Coyle 1979; Clarke et al. 1997).

Whole brains (except cerebellum) are obtained from male Wistar rats. Fifteen mg of a membrane fraction prepared by standard techniques is incubated with 5.0 nM [^{3}H]kainate for 1 h at 4 °C. Non-specific binding is estimated in the presence of 1 mM L-glutamate. Membranes are filtered and washed 3 times to separate free from bound ligand and filters are counted to determine [^{3}H]kainate bound.

Toms et al. (1997), Zhou et al. (1997) recommended [^{3}H]-(2S,4R)-4-methylglutamate as kainate-receptor selective ligand.

Irreversible inhibition of high affinity [^{3}H]kainate binding by a photoactivatable analogue was reported by Willis et al. (1997).

Worms et al. (1981) described the behavioral effects of systemically administered kainic acid.

Glutamate NMDA-agonist site

The assay measures the binding of CGS 19755, a selective antagonist, to the agonist site of the NMDA receptor (Lehmann et al. 1988; Murphy et al. 1988; Jones et al. 1989)

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E.3.1.7 NMDA receptor complex: [^{3}H]TCP binding

PURPOSE AND RATIONALE

The purpose of this assay is to determine the binding affinity of potential noncompetitive NMDA antagonists at the phencyclidine (PCP) binding site which is believed to be within or near the NMDA-regulated ion channel. TCP, 1-[1-(2-thienyl)cyclohexyl]-piperidine, is a thienyl derivative of PCP.

Excessive activity of excitatory amino acid neurotransmitters has been associated with the neuropatholo-

gies of epilepsy, stroke and other neurodegenerative disorders (Cotman and Iversen 1987; Watkins and Olverman 1987). Antagonism of NMDA receptor function appears to be the mechanism of action of some novel anticonvulsant and neuroprotective agents. Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA) and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987). The binding site for [^{3}H]2-amino-4-phosphonobutyric acid (AP4) may represent a fourth site which is less well characterized. NMDA receptors are believed to be coupled to a cation channel which converts to an open state following activation (Kemp et al. 1987). The opening and closing of this cation channel are also modulated by glycine, Mg^{2+}, Zn^{2+} and polyamines (Loo et al. 1986; Snell et al. 1987; Reynolds et al. 1988; Thomson 1989; Snell et al. 1988; Sacaan and Johnson 1989; Thedinga et al. 1989; Williams et al. 1989). Dissociative anesthetics, such as phencyclidine (PCP) and ketamine, and the neuroprotective agent MK-801 block the ion channel and are noncompetitive NMDA receptor antagonists. Competitive NMDA receptor antagonists, such as 3-[(±)-2-carboxypiperazin-4-yl]-1-phosphonic acid (CPP), and the phosphono analogs of L-glutamate, 2-amino-7-phosphonoheptanoic acid (2-AP7) and 2-amino-5-phosphonopentanoic acid (2-AP5), are inhibitors at the excitatory amino acid recognition site.

Molecular cloning and functional expression of rat and mouse NMDA receptors (Moriyoshi et al. 1991; Meguro et al. 1992), a family of AMPA-selective glutamate receptors (Keinänen et al. 1990) and the metabotropic glutamate receptors mGluR1–mGluR6 (Schoepp et al. 1990; Masu et al. 1991; Abe et al. 1992; Bashir et al. 1993; Nakajima et al. 1993; Tanabe et al. 1993) have been reported.

PROCEDURE I

Reagents

1. Buffer A: 0.1 M HEPES, pH 7.5
   Weigh 23.83 g HEPES
   Add approximately 900 ml distilled water.
   Adjust pH to 7.5 with 10 N NaOH.
   q.s. to 1 liter with distilled water.
2. Buffer B: 10 mM HEPES, pH 7.5
   Dilute buffer A 1 : 10 with distilled water and adjust pH to 7.5.
3. L-Glutamic acid, 5×10^{-3} M
   Dissolve 7.36 mg with 10.0 ml distilled water.
   Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-4} M.
4. Glycine, 5×10^{-4} M
   Dissolve 3.75 mg with 10.0 ml distilled water.
   Dilute 1 : 10 with distilled water.
   Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-5} M.
5. Phencyclidine HCl (PCP) is used for nonspecific binding.
   Dissolve 0.7 mg in 0.5 ml distilled water.
   Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-4} M.
6. [^{3}H]TCP is obtained from New England Nuclear, specific activity 42–60 Ci/mmole. For IC_{50} determinations, a 50 nM stock solution is made with distilled water. Aliquots of 50 µl are added to each tube to yield a final concentration of 2.5 nM.
7. Test compounds. A stock solution of 5 mM is made up with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.

Tissue preparation

Cerebral cortex of male Wistar rats, 7–10 weeks of age, is dissected over ice and homogenized in ice-cold 0.32 M sucrose, 30 volumes of original tissue weight, for 60 s with a Tissumizer setting at 70. The homogenate is centrifuged at 1 000 *g* for 10 min. (SS34, 3 000 rpm, 4 °C). The supernatant is centrifuged at 20 000 *g* for 20 min. (SS34, 12 000 rpm, 4 °C). The pellet is resuspended with cold distilled water, to 50 volumes of original tissue weight, using the Tissumizer, 60 s at setting of 70. The homogenate is incubated at 37 °C for 30 min, transferred to centrifuge tubes, and centrifuged at 36 000 *g* for 20 min. (SS34, 16 500 rpm, 4 °C). The pellet is again resuspended in 50 volumes distilled water, incubated and centrifuged. All resuspensions with the Tissumizer are for 60 s at a setting of 70. The resulting pellet is resuspended in 30 volumes of ice-cold 10 mM HEPES buffer, pH 7.5, centrifuged, and washed once again (resuspension and centrifugation) with buffer. Following resuspension in 30 volumes of buffer, the homogenate is frozen in the centrifuge tube and stored at –70 °C until the day of the assay.

On the day of the assay, the homogenate is thawed and centrifuged at 36 000 *g* for 20 min. (SS34, 16 500 rpm, 4 °C). The pellet is washed three times by resuspension with ice-cold 10 mM HEPES buffer, pH 7.5, centrifuged and finally resuspended in 30 volumes of buffer. Aliquots of 500 µl are used for each assay tube, final volume 1 000 µl, and correspond to approximately 0.2 mg protein.

Assay

1. Prepare assay tubes in triplicate. For each test compound, inhibition of [^{3}H]TCP binding is measured

both in the absence (basal) and presence (stimulated) of 100 µM L-glutamic acid and 10 mM glycine.

Basal	Stimulated	
380 µl	340 µl	Distilled water
50 µl	50 µl	Buffer A, 0.1 M HEPES, pH 7.5
20 µl	20 µl	PCP (reagent A5) or distilled water, or appropriate concentration of inhibitor
0 µl	20 µl	L-glutamic acid (reagent A3)
0 µl	20 µl	Glycine (reagent A4)
50 µl	50 µl	[^{3}H]TCP (reagent A6)
500 µl	500 µl	Tissue homogenate

2. Following the addition of the tissue, the tubes are incubated for 120 min at 25 °C with agitation. The assay is terminated by separating the bound from non-bound radioligand by rapid filtration with reduced pressure over Whatman GF/B filters, presoaked in 0.05% polyethylene-imine, using the Brandell cell harvesters. The filters are rinsed once with buffer before filtering the tubes, and rinsed two times after filtration. The filters are counted with 10 ml Liquiscint.

EVALUATION

Specific binding is determined from the difference of binding in the absence or presence of 10^{-4} M PCP. Specific binding is typically 50% of total binding in basal conditions, and 90% of total binding when stimulated by L-glutamic acid and glycine. L-Glutamic acid and glycine typically increase specific binding to 300% and 200% of basal binding, respectively. The combination of L-glutamic acid and glycine typically produce a greater than additive effect, increasing specific binding to 700% of basal binding. IC_{50} values for the competing drug are calculated by log-probit analysis of the data.

Protocol modification for crude membrane homogenates

This modified procedure for the preparation of membrane homogenates does not use extensive lysing and washing of the tissue to remove endogenous L-glutamate, glycine and other endogenous compounds which enhance [^{3}H]TCP binding. This procedure may be used for rapid screening of compounds for inhibition of [^{3}H]TCP binding site without specifically defining an interaction at the ion channel or modulatory sites of the NMDA receptor complex.

PROCEDURE II

Reagents

1. Buffers A and B are prepared as described above.
2. Phencyclidine HCl is used for nonspecific binding and is prepared as described above.
3. [^{3}H]TCP is prepared as described above.
4. Test compounds are prepared as described above.

Tissue preparation

Cortical tissue is dissected, homogenized in 30 volumes of 0.32 M sucrose and a crude P_2 pellet is prepared as described above. The pellet is resuspended in 30 volumes of 10 mM HEPES, pH 7.5, centrifuged at 36 000 *g* (SS34, 16 500 rpm, 4 °C) for 20 min, and again resuspended in 100 volumes of buffer. This homogenate is used directly in the assay in aliquots of 500 µl.

Assay

1. Prepare assay tubes in triplicate.

Volume	Solution
380 µl	Distilled water
50 µl	Buffer A, 0.1 M HEPES, pH 7.5
20 µl	PCP (reagent A5) or distilled water, appropriate concentration of inhibitor
50 µl	[^{3}H]TCP (reagent A6)
500 µl	Tissue homogenate

2. Following the addition of the tissue, the tubes are incubated for 120 min at 25 °C with agitation. The assay is terminated by rapid filtration as described above. The filters are rinsed and counted for bound radioactivity as above.

EVALUATION

Specific binding is determined from the difference of binding in the presence or absence of 10^{-4} M PCP. Specific binding is typically 90% of total binding. IC_{50} values for the competing drug are calculated by log-probit analysis.

MODIFICATIONS OF THE METHOD

Instead of [^{3}H]TCP, radiolabeled [^{3}H]MK-801 has been used as ligand (Wong et al. 1988; Javitt and Zukin 1989; Williams et al. 1989).

Sills et al. (1991) described [^{3}H]CGP 39 653 as a N-methyl-D-aspartate antagonist radioligand with low nanomolar affinity in rat brain.

Nowak et al. (1995) reported that swim stress increases the potency of glycine to displace 5,7-[^{3}H[di-

chlorokynurenic acid from the strychnine-insensitive glycine recognition site of the *N*-methyl-D-aspartate receptor complex.

NMDA receptor cloning studies have shown that NMDA receptors contain at least one of seven different NMDAR1 subunits (NR1A–NR1G) (Sugihara et al. 1992) and at least one of four NMDAR2 subunits (NR2A–NR2D) (Kutsuwada et al. 1992; Ishii et al. 1993). While the NR1 subunits are generated by alternative splicing of a single gene, the NR2 subunits are products of four highly homologous genes. Thus, there are thousands of potential subunit combinations yielding complexes of four or five subunits.

Grimwood et al. (1996) reported generation and expression of stable cell lines expressing recombinant human NMDA receptor subtypes, two cell lines expressing NR1a/NR2A receptors and one cell line expressing NR1a/NR2B receptors.

NR2B selective NMDA antagonists were described by Fischer et al. (1997), Kew et al. (1998), Reyes et al. (1998), Chenard and Menniti (1999).

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E.3.1.8 Metabotropic glutamate receptors

PURPOSE AND RATIONALE

In addition to ionotropic (AMPA, kainate and NMDA) receptors, glutamate interacts with a second family of receptors, metabotropic or mGlu receptors (Tanabe et al. 1992, 1993; Schoepp and Conn 1993; Hollmann and Heinemann 1994; Nakanishi and Masu 1994; Okamoto et al. 1994; Watkins and Collingridge 1994; Knöpfel et al. 1995, 1996; Pin and Duvoisin 1995; Conn and Pin 1997; Alexander et al. 2001; Skerry and Generver 2001; DeBlasi et al. 2001). Three groups of native receptors are distinguishable on the basis of similarities of agonist pharmacology, primary sequence, and G protein-effector coupling: Group I ($mglu_1$ and $mglu_5$ and splice variants) are coupled via $G_{q/11}$ to phosphoinositide hydrolysis. Group II ($mglu_2$ and $mglu_3$) are negatively coupled via G_i/G_o to adenylyl cyclase and inhibit the formation of cAMP following exposure of cells to forskolin or activation of an intrinsic G_s-coupled receptor (e.g. adenosine A_2 receptor). The group III receptors ($mglu_4$, $mglu_6$, $mglu_7$, and $mglu_8$) also inhibit forskolin-stimulated adenylyl cyclase.

Various agonists and antagonists for metabotropic glutamate receptors were described (Ishida et al. 1990, 1994; Porter et al. 1992; Jane et al. 1994; Watkins and Collingridge 1994; Knöpfel et al. 1995; Annoura et al. 1996; Bedingfield et al. 1996; Thomsen et al. 1996; Acher et al. 1997; Doherty et al. 1997; Brauner-Osborne et al. 1998; Kingston et al. 1998; Monn et al. 1999; Jane and Doherty 2000).

Several radioligands for metabotropic glutamate receptors were described:

- for subtype mGluR4a receptor by Eriksen and Thomsen (1995),
- for group II mGlu receptors by Cartmell et al. (1998), by Ornstein et al. (1998), and by Schaffhauser et al. (1998).

Riedel and Reymann (1996) discussed the role of metabotropic glutamate receptors in hippocampal long-term potentiation and long-term depression and their importance for learning and memory. Furthermore, possible roles in the treatment of neurodegenerative disorders (Nicoletti et al. 1996; Bruno et al. 1998), and of Parkinson's disease (Konieczny et al. 1998) were discussed. Anticonvulsive properties (Attwell et al. 1998; Thompson and Dalby 1998; Gasparini et al. 1999) as well as anxiolytic properties (Helton et al. 1998) of metabotropic glutamate receptor ligands were reported. Christoffersen et al. (1999) found a positive effect on short-term memory and a negative effect on long-term memory of the class I metabotropic glutamate receptor antagonist, AIDA, in rats.

PROCEDURE

Cultured cells are prepared from cerebral cortex of 17-day embryos of Wistar rats. Prior to the experiments, the culture is maintained for 8–12 days with minimum essential medium (MEM) containing 5% fetal calf serum and 5% horse serum.

For **cyclic AMP assays**, the cultured cells are preincubated with HEPES-buffered Krebs-Ringer solution containing 5.5 mM glucose (HKR) for 1–1.5 h, then exposed to various agonists for 15 min in the absence or presence of 10 μM forskolin. The content of cyclic AMP is measured using a radioimmuno-assay kit after homogenization with 0.1 M HCl.

For **phosphoinositide turnover assays**, the cultured cells are prelabeled with myo-1,2-[^{3}H]inositol in MEM for 8–10 h. The cells are washed twice with HKR containing 10 mM LiCl, and then exposed to various agonists in HKR containing 10 mM LiCl for 30 min. The reaction is terminated with 2% trichloroacetic acid, and the homogenized samples are analyzed for inositol constituents by anion exchange chromatography (Berridge et al. 1982). The extracts are applied to columns containing 1 ml of Dowex 1 in the formate form. The phosphate esters are then eluted by the step-wise addition of solutions containing increasing concentrations of formate. Glycerophosphoinositol and inositol 1 : 2-cyclic phosphate are eluted with 5 mM-sodium tetraborate plus 150 mM sodium formate. The penultimate solution contains 0.1 M-formic acid plus 0.3 M-ammonium formate, followed by 0.1 M-formic acid plus 0.75 M-ammonium formate, each of which removes more polar inositol phosphates.The 1 ml fractions eluted from the columns are count-ed for radioactivity after addition of 10 ml of Biofluor.

The percentage of radioactivity of inositol phosphates to the total applied to the column is calculated.

EVALUATION

Dose-response curves for inhibition of forskolin-stimulated cAMP formation and for percentage of phosphoinositide hydrolysis are established for each test compound.

MODIFICATIONS OF THE METHOD

Thomsen et al. (1993, 1994) used baby hamster kidney (BHK) cells stably expressing $mGluR_{1\alpha}$, $mGluR_2$ or $mGluR_4$ for measurements of phosphoinositol hydrolysis or cAMP formation.

Varney and Suto (2000) recommended functional high throughput screening assay for the discovery of subtype-selective metabotropic glutamate receptor ligands.

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E.3.1.9 Excitatory amino acid transporters

PURPOSE AND RATIONALE

Glutamate is not only the predominant excitatory neurotransmitter in the brain but also a potent neurotoxin. Following release of glutamate from presynaptic vesicles into the synapse and activation of a variety of ionotropic and metabotropic glutamate receptors, glutamate is removed from the synapse. This is achieved through active uptake of glutamate by transporters located prebut also post-synaptically, or glutamate can diffuse out of the synapse and be taken up by transporters located on the cell surface of glial cells. The excitatory amino acid transporters form a gene family out of which at least 5 subtypes were identified (Robinson et al. 1993; Seal and Amara 1999). A role for glutamate transporters has been postulated for acute conditions such as stroke, CNS ischemia, and seizure, as well as in chronic neurodegenerative diseases, such as Alzheimer's disease and amyotrophic lateral sclerosis. Glutamate transport is coupled to sodium, potassium and pH gradients across the cell membrane creating an electrogenic process. This allows transport to be measured using electrophysiological techniques (Vandenberg et al. 1997).

PROCEDURE

Complementary DNAs encoding the human glutamate transporters, EAAT1 and EAAT2, are subcloned into pOTV for expression in X. laevis oocytes (Arriza et al. 1994; Vandenberg et al. 1995). The plasmids are linearized with BAMHI and cRNA is transcribed from each of the cDNA constructs with T7 RNA polymerase and capped with 5',7-methyl guanosine using the mMESSAGE mMACHINE (Ambion, Austin TX). cRNA (50 ng) encoding either EAAT1 or EAAT2 is injected into defolliculated Stage V X. laevis oocytes. Two to 7 days later, transport is measured by two-electrode voltage-clamp recording using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) and a MacLab 2e recorder (ADI Instruments, Sydney, Australia) and controlled using a pCLAMP 6.01 interfaced to a Digidata 1 200 (Axon Instruments). Oocytes are voltage-clamped at –60 mV and continuously superfused with ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.5). For transport measurement, this buffer is changed to one containing the indicated concentration of substrate and/or blocker. The voltage dependence of block of glutamate transport is measured by clamping the membrane potential at –30 mV and then applying a series of 100 ms voltage pulses from –100 mV to 0 mV and measuring the steady state current at each membrane potential. This protocol is applied both before and during the application of the compound in question and then the base-line current at each membrane potential is subtracted from the current in the presence of the compounds to get a measure for the transport specific current at the various membrane potentials.

EVALUATION

Current (I) as a function of substrate concentration ([S]) is fitted by least squares to

$$I = I_{max}\,[S] / (K_m + [S])$$

where I_{max} is the maximal current and K_m is the Michaelis transport constant. The I_{max} values for the various substrates are expressed relative to the current generated by a maximal dose of L-glutamate in the same cell. I_{max} and K_m values are expressed as mean ± standard error and are determined by fitting data from individual oocytes. The potent competitive blockers are characterized by Schild analysis (Arunlakshana and Schild 1959) and the K_b estimated from the regression plot. The less potent blockers are assumed to be competitive and K_i values calculated from IC_{50} values using the equation

$$K_i = IC_{50} / (1 + [\text{glutamate}] / K_m)$$

where K_i is the inhibition constant, IC_{50} is the concentration giving half maximum inhibition, K_m is the transport constant and [glutamate] is 30 μM. The fraction of the membrane electric field sensed by transport blockers when bound to the transporters is estimated using the Woodhull equation (Woodhull 1973),

$$K_i = K_i^0 \exp(-\zeta\,\delta\,\mathrm{F}\,E / \mathrm{R}\,T)$$

where K_i is the inhibition constant, K_i^0 is the inhibition constant at 0 mV, ζ is the charge on the blocker, δ is the fraction of the membrane field, F is Faraday's constant, E is the membrane potential, R is the gas constant and T is temperature in °K.

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E.3.1.10 [^{35}S]TBPS binding in rat cortical homogenates and sections

PURPOSE AND RATIONALE

To screen potential anticonvulsant agents which interact at the convulsant binding site of the benzodiazepine/GABA/chloride ionophore complex by measuring the inhibition of binding of [^{35}S]TBPS to rat cortical membranes.

TBPS, t-butylbicyclophosphorothionate, is a potent convulsant which blocks GABA-ergic neurotransmission by interacting with the convulsant (or picrotoxin) site of the GABA/benzodiazepine/chloride ionophore receptor complex (Casida et al. 1985; Gee et al. 1986; Olsen et al. 1986; Squires et al. 1983; Supavilai and Karabath 1984). Picrotoxin, pentylenetetrazol and the so-called "cage convulsants" are believed to change the state of the chloride channel to a closed conformation, and thereby block GABA-induced increases in chloride permeability. Anticonvulsants such as the barbiturates and the pyrazolopyridines, cartazolate, etazolate and tracazolate, appear to interact at depressant sites allosterically coupled to the convulsant sites, and facilitate the effects of GABA on chloride permeability, by converting the ionophore to the open conformation. Benzodiazepines interact at a separate recognition site to modulate the actions of GABA. Convulsant compounds and some anticonvulsants can inhibit [^{35}S]TBPS binding. These two classes can be differentiated by their effects on dissociation kinetics (Macksay and Ticku 1985; Trifiletti et al. 1984, 1985). [^{35}S]TBPS dissociates slowly, half-life approximately 70 min, in a monophasic manner in the presence of convulsant compounds; anticonvulsants produce a biphasic dissociation, with rapid and slow phase components. It has been postulated that the rapid and slow phases of [^{35}S]TBPS dissociation may correspond to the open and closed conformation of the chloride ionophore.

PROCEDURE

Reagents

1. Buffer A 0.05 M Tris with 2 M KCl, pH 7.4
   6.61 g Tris HCl
   0.97 g Tris Base
   149.1 g KCl
   q.s. to 1 liter with distilled water
2. Buffer B
   a 1 : 10 dilution of buffer A in distilled water (5 mM Tris, 200 mM KCl, pH 7.4)
3. [^{35}S]TBPS is obtained from New England Nuclear with a high initial specific activity, 90–110 Ci/mmol. For an inhibition assay with a 2 nM final concentration of TBPS, a specific activity of 20–25 Ci/mmol will provide sufficient counts due to a high counting efficiency (87%) for ^{35}S. The specific activity of [^{35}S]TBPS can be reduced with the addition of 3–5 vol-umes (accurate measurement with a Hamilton syringe) of an equimolar ethanolic solution of non-radiolabeled TBPS (7.9×10^{-6} M). The new specific activity (Ci/mmol) is calculated by dividing the number of Curies by the number of mmoles TBPS. Since [^{35}S]TBPS has a relatively short half-life, 87.1 days, the specific activity is calculated for each assay, based on the exponential rate of decay:

   A_0 = initial specific activity
   A = specific activity at time t
   t = days from date of initial calibration of specific activity
   $t_{1/2}$ = half-life of [^{35}S] in days (87.1)

   For IC_{50} determinations, a 40 nM stock solution is made with distilled water and 25 µl is added to each tube to yield a final concentration of 2 nM in the assay.
4. Unlabeled TBPS is available from New England Nuclear. A stock dilution of 7.923×10^{-6} M is prepared in ethanol.
5. Picrotoxin, is obtained from Aldrich Chemical Company. A solution of 5×10^{-4} M is prepared with distilled water, with sonication if necessary. Aliquots of 10 µl are added to assay tubes to give a final concentration of 10^{-5} M.
6. Test compounds. A stock solution of 1 mM is made up with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.

Tissue preparation

Whole cerebral cortex of male Wistar rats is dissected over ice and homogenized with a Tekmar Tissumizer, 20 s at setting 40, in 20 volumes of 0.32 M sucrose, ice-cold. The homogenate is centrifuged at 1 000 *g* for 10 min (SS34, 3 000 rpm, 4 °C). The supernatant is then centrifuged at 40 000 *g* for 30 min (SS34, 20 000 rpm, 4 °C). The resulting pellet is resuspended in 20 volumes of ice-cold distilled water with two 6-s bursts of the Tissumizer, setting 40. The homogenate is centrifuged at 40 000 *g* for 30 min. The pellet is washed (resuspended and centrifuged) once with 20 volumes ice-cold buffer (Tris HCl 5 mM, KCl 200 mM, pH 7.4). The resulting pellet is resuspended with 20 volumes buffer and frozen at –70 °C overnight. The following day, the tissue homogenate is thawed in a beaker of warm water, approximately 15 min, and then centrifuged at 40 000 *g* for 30 min (SS34, 20 000 rpm, 4 °C). The pellet is washed twice with 20 volumes of ice-cold buffer, and then resuspended and frozen at –70 °C for future use. On the day of the assay, the homogenate is thawed and centrifuged at 40 000 *g* for 30 min. The resulting pellet is washed once with 20 volumes ice-cold buffer and finally resuspended in 30 volumes buffer. Aliquots of 250 µl are used for each assay tube, final volume 500 µl, and correspond to 8.35 mg original wet weight tissue per tube, approximately 0.2 mg protein.

Assay

1. Prepare assay tubes in triplicate:
   - 190 µl distilled water
   - 25 µl Tris 0.05 M, KCl 2M, pH 7.4
   - 10 µl picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor 25 µl [^{35}S]-TBPS, final concentration 2 nM
   - 250 µl tissue preparation, 1 : 30 homogenate
2. Following the addition of the tissue, the tubes are incubated at 25 °C for 150 min with agitation. The assay is terminated by rapid filtration over Whatman GF/B filter circles, presoaked in buffer, with 5 × 4 ml rinses of ice-cold buffer. Vacuum filtration is performed with the 45-well filtration units to avoid contamination of the Brandell harvesters with [^{35}S]. The filters are counted with 10 ml Liquiscint.

EVALUATION

Specific binding is determined from the difference between binding in the absence or presence of 10 mM picrotoxin, and is typically 85–90% of total binding. The percent inhibition at each drug concentration is the mean of triplicate determinations. IC_{50} values for the competing drug are calculated by log-probit analysis of the data.

Modifications for dissociation experiments

1. Prepare assay tubes as follows:
   - 185 µl distilled water
   - 25 µl Tris 50 mM, KCl 2 M, pH 7.4
   - 10 µl test compound or vehicle
2. Add 250 ml tissue homogenate to tubes. Vortex. Preincubate 30 min at 25 °C.
3. Add 25 ml [^{35}S]-TBPS. Vortex. Incubate 180 min at 25 °C.
4. Add 5 ml picrotoxin (10^{-3} M) to give a final concentration of 10^{-5} M. Vortex.
5. At various times after the addition of picrotoxin (0–120 min) tubes are filtered and rinsed as described above.

Modification for [^{35}S]-TBPS autoradiography

1. Sections of rat brain, 20 mm thickness, are collected onto gel-chrome alum subbed slides, freeze-dried for approximately 1 h and stored at –70 °C until used.
2. After thawing and drying at room temperature, the sections are preincubated for 30 min in buffer B.
3. Preparation of slide mailers for incubation:
   a) for scintillation counting:
      - 2.47 ml distilled water
      - 0.325 ml buffer A
      - 3.25 ml buffer B
      - 0.13 ml picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor
      - 0.325 ml [^{35}S]TBPS, final concentration 2 nM
      - 6.50 ml final volume

   b) for autoradiography:
      - 4.56 ml distilled water
      - 0.60 ml buffer A
      - 6.00 ml buffer B
      - 0.24 ml picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor
      - 0.60 ml [^{35}S]TBPS, final concentration 2 nM
      - 12.0 ml final volume
4. Sections are incubated in slide mailers at room temperature with [^{35}S]TBPS in the absence or presence of appropriate inhibitors for 90 min.
5. Slides are transferred to vertical slide holders and rinsed in ice-cold solutions as follows: dip in buffer B, two 5 minute rinses in buffer A and a dip in distilled water.
6. Slides are dried under a stream of cool air and desiccated overnight at room temperature.
7. Slides are mounted onto boards with appropriate [^{35}S] brain mash standards.
8. In the dark room under safelight illumination (GBX filter), slides are opposed to Kodak X-OMAT AR film and stored in cassettes for 7–10 days.
9. Develop films as described in "X-OMAT AR Film Processing".

	[^{35}S]TBPS binding parameters	
	Slide-mounted sections	**Cortical homogenates**
Assay conditions		
Tissue	20 µ sections, rat freeze-dried, 1 h	Whole cortex, rat 1:30 homogenate prepared with 5 washes and 2 freeze-thaw cycles
	30 min preincubation	No preincubation
Buffer	5 mM Tris 200 mM KCl pH 7.4	5 mM Tris 200 mM KCl pH 7.4
Incubation time	90 min 21–22 ˚C	150 min 25 ˚C
Non specific	10^{-5} M picrotoxin	10^{-5} M picrotoxin
Tissue linearity	2.5–25 mg tissue per 0.5 ml assay tube	
Equilibrium constants		
*K*d (nM)	32.8	25.2
*B*max (fmol/mg prot)	1615	2020
Binding kinetics		
Association kobs (min^{-1})	0.0496	0.0138
$k+1$ (nM^{-min-1})	0.0164	0.0021
Dissociation $k-1$ (min^{-1})	0.017	0.001
Dissociation constant $k+1 / k-1$ (nM)	1.03	4.73
IC_{50} M		
Picrotoxin	2.8×10^{-7}	3.4×10^{-7}
TBPS	8.7×10^{-8}	8.1×10^{-8}
GABA	1.7×10^{-6}	2.1×10^{-6}
Pentobarbital	1.2×10^{-4}	6.0×10^{-4}
Phenobarbital	None at 10^{-3}	None at 10^{-3}
Clonazepam	None at 10^{-6}	None at 10^{-6}

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E.3.1.11 [^{3}H]glycine binding in rat cerebral cortex

PURPOSE AND RATIONALE

This assay is used to assess the affinity of compounds for the glycine binding site associated with the N-methyl-D-aspartate (NMDA) receptor complex using [^{3}H] glycine as the radioligand.

The amino acid glycine modulates and may activate the excitatory amino acid receptors of the NMDA-subtype (Thomson 1989). Glycine has been shown *in vitro* to potentiate the effects of l-glutamate or NMDA on the stimulation of [^{3}H]TCP binding (Snell et al. 1987, 1988; Bonhaus 1989) and [^{3}H]norepinephrine release (Ransom et al. 1988), and *in vivo* to act as a positive modulator of the glutamate-activated cGMP response in the cerebellum (Danysz et al. 1989; Rao et al. 1990). The activation of NMDA receptors requiring the presence of glycine is necessary for the induction of long-term potentiation (LTP), a type of synaptic plasticity which may be fundamental to learning processes (Oliver et al. 1990). A [^{3}H]glycine binding site in the brain has been identified and characterized as a strychnine-insensitive site associated with the NMDA receptor complex (Kessler et al. 1989; Monahan et al. 1989; Cotman et al. 1987). Autoradiographic studies have shown a similar distribution of [^{3}H]glycine and [^{3}H]TCP (NMDA ion channel radioligand) binding sites (Jansen et al. 1989). Compounds which interact with the glycine site offer a novel mechanism of action for intervention with NMDA receptor function.

PROCEDURE

Reagents

1. Buffer A: 0.5M Tris maleate, pH 7.4
   59.3 g Tris maleate
   q.s. to 0.5l
   Adjust pH to 7.4 with 0.5M Tris base.
2. Buffer B: 50 mM Tris maleate, pH 7.4
   Dilute buffer A 1 : 10 with distilled water; adjust pH with 50 mM Tris maleate (acid) or 50 mM Tris base.
3. Glycine, 5×10^{-2} M
   Dissolve 3.755 mg of glycine (Sigma G7 126) with 1.0 ml distilled water. Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-3} M
4. [^{3}H]Glycine is obtained from New England Nuclear, specific activity 45–50 Ci mmol. For IC_{50} determinations, a 200 nM stock solution is made with distilled water. Aliquots of 50µl are added to yield a final assay concentration of 10 nM.
5. Test compounds. A stock solution of 5 mM is prepared with a suitable solvent and serially diluted, such that the final concentrations in the assay ranges from 10^{-4} to 10^{-7} M. Higher or lower concentrations may be used, depending on the potency of the compound.
6. Triton-X 100, 10% (v/v) (National Diagnostics, EC-606). A stock solution of Triton-X 100, 10% can be prepared and stored in the refrigerator. Dilute 1.0 ml of Triton-X 100 to 10.0 ml with distilled water. On the day of the assay, the tissue homogenate (1 : 15 dilution) is preincubated with an aliquot of the 10% solution to give a final concentration of 0.04% (v/v).

Tissue preparation

Cortices of male Wistar rats are dissected over ice and homogenized in ice-cold 0.32 M sucrose, 15 volumes of original wet weight of tissue, for 30 s with a Tissumizer setting at 70. Three cortices are pooled for one preparation. The homogenate is centrifuged at 1 000 *g* for 10 min (SS34, 3 000 rpm, 4 °C). The supernatant is centrifuged at 20 000 *g* (SS34, 12 000 rpm, 4 °C) for 20 min. Resuspend the pellet in 15 volumes of ice-cold distilled water (Tissumizer setting 70, 15 s) and spin at 7 600 *g* (SS34, 8 000 rpm 4 °C) for 20 min. The pellet is resuspended with 15 volumes of cold distilled water and centrifuged. Discard the supernatant and store the pellet at –70 °C.

On the day of the assay, the pellet is resuspended in 15 volumes ice-cold 50 mM Tris maleate, pH 7.4. Preincubate the homogenate with Triton-X in a final concentration of 0.04% (v/v) for 30 min at 37 °C with agitation. Centrifuge the suspension at 48 000 *g* (SS34, 20 000 rpm, 4 °C) for 20 min. Wash the pellet an additional 3 times by resuspension with cold buffer and centrifugation. The final pellet is resuspended in a volume 25 times the original wet weight.

Assay

1. Prepare assay tubes in quadruplicate.
   380 µl distilled water
   50 µl buffer A, 0.5 M Tris maleate, pH 7.4
   20 µl glycine, 10^{-3} M final concentration, or distilled water or appropriate concentration of inhibitor
   50 µl [^{3}H] glycine, final concentration 10 nM
   500 µl tissue homogenate.
   1 000 µl final volume
2. Following the addition of the tissue, the tubes are incubated for 20 min in an ice-bath at 0–4 °C. The binding is terminated by centrifugation (HS4, 7 000 rpm, 4 °C) for 20 min. Aspirate and discard the superna-tant. Carefully rinse the pellet twice with 1 ml ice-cold buffer, avoiding disruption of the pellet. Transfer the pellet to scintillation vials by vor-texing the pellet with 2 ml scintillation fluid, rinse the tubes twice with 2 ml and add an additional 4 ml scintillation fluid.

EVALUATION

Specific binding is determined from the difference of binding in the absence or in the presence of 10^{-4} M glycine and is typically 60–70% of total binding. IC_{50} values for the competing compound are calculated by log-probit analysis of the data.

MODIFICATIONS OF THE METHOD

Baron et al. (1996), Hofner and Wanner (1997), Chazot et al. (1998) described [^{3}H]MDL 105,519 as a high affinity ligand for the NMDA associated glycine recognition site.

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E.3.1.12 [^{3}H]strychnine sensitive glycine receptor

PURPOSE AND RATIONALE

The strychnine-sensitive glycine receptor is a member of the family of ligand-gated ion channel receptors. Within this family, the glycine receptor is most closely related to the GABA-receptor. Like the $GABA_A$-receptor, the glycine receptor has an inhibitory role, mediating an increase in chloride conductance. However, in contrast to the $GABA_A$-receptor, the glycine receptor is located mainly in the spinal cord and lower brainstem, where glycine appears to be the major inhibitory neurotransmitter. Purification and molecular cloning has shown that the glycine receptor is an oligomeric transmembrane protein complex composed of three α and two β subunits. The inhibitory actions of glycine are potently blocked by strychnine. In addition to strychnine, the steroid derivative RU5 135 (Simmonds and Turner 1985), phenylbenzene-ω-phosphono-α-amino acid (Saitoh et al. 1996), and 5,7-dichloro-4-hydroxyquinoline-3-carboxylic acid (Schmieden et al. 1996) antagonize glycine responses in cultured neurons or cells expressing recombinant glycine receptors.

A glycine receptor agonist may be a potential antispastic agent.

PROCEDURE

Male Wistar rats weighing about 200 g are sacrificed. About 220 mg of frozen pons and medulla are homogenized in 2×10 ml ice-cold 50 mM potassium phosphate buffer, pH 7.1, by an Ultra-Turrax homogenizer. The homogenate is centrifuged for 10 min at 30 000 *g* at 0–4 °C in a refrigerated centrifuge. The pellet is rehomogenized in another 2×10 ml portion of the same buffer and recentrifuged as before. This washing procedure is repeated a total of four times. The final pellet is resuspended in 200 vol/g original tissue in ice-cold 50 mM potassium phosphate buffer, pH 7.1, with or without 1 000 mM NaCl, and used directly for binding assays.

Binding assays consist of 1 ml tissue homogenate, 50 µl test solution (water or 5% v/v ethanol/water is used for serial dilutions), 50 µl water, 5% ethanol/water or glycine solution (40 mM final concentration), and 25 µl [^{3}H]strychnine working solution, final concentration 2 nM. The samples are mixed well and incubated for 20 min in an ice bath. Free and bound radioactivity are separated by filtration through Whatman GF/C glass fiber filters followed by washing with 2×10 ml ice-cold 50 mM potassium phosphate buffer, pH 7.1. Tritium on the filters is monitored by convential scintillation counting in 3 ml Hydroluma. Nonspecific binding is binding in the presence of 40 mM glycine and is

always subtracted from total binding to give specific binding.

EVALUATION

K_i values are calculated as

$$K_i = IC_{50} / (1 + [K_D] / [L])$$

whereby: IC_{50} are the concentrations that inhibit by 50% the specific binding of [^{3}H]strychnine determined in two independent experiments using at least 3 concentrations of the agent in duplicate assays, [L] is the concentration of the radioligand, K_D is the affinity constant in the absence or the presence of 1 000 mM NaCl.

NaCl shift used for differentiating glycine agonists from glycine antagonists is the ratio K_i 1 000 mM NaCl versus K_i 0 mM NaCl.

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E.3.1.13 Electrical recordings from hippocampal slices *in vitro*

PURPOSE AND RATIONALE

The transverse hippocampal slice has been described as a well-defined cortical structure maintained *in vitro* (Skrede and Westgard 1971). The hippocampus slice has the advantage that each slice may contain all hippocampal structures: The chain of neurons goes from the perforant path to granule cells of the dentate gyrus, through mossy fibres to CA-3 pyramidal cells and then through Schaffer collaterals to CA-1 cells with their axons leaving the hippocampus through the alveus. The pyramidal cells lie close together and can be easily seen and penetrated with fine microelectrodes.

PROCEDURE

Male guinea pigs weighing 300–400 g are anesthetized with ether, the brains removed and the hippocampi dissected. Transverse slices of the hippocampus (300–400 µm thick) are cut in parallel to the alvear fibres. After preparation, the slices are submerged in 28 °C warm saline which is equilibrated with 95% O_2 and 5% CO_2. After a preincubation period of 2 h, slices are transferred in a Perspex chamber (1.5 × 4 cm) and attached to the bottom consisting of optically plain glass. The chamber is mounted on an inverted microscope allowing detailed inspection of the excised tissue. The slices are superfused by an approximately 3 mm thick layer of 32 °C warm saline. Intracellular recordings are achieved by means of micropipettes with tip diameters of less than 0.5 µm which are filled with 3 mol/l potassium chloride. Under microscopic control the tips of the micropipettes are placed within the stratum pyramidale and moved by means of a step motor driven hydraulic microdrive. For intracellular injections of drugs, e.g., pentylenetetrazol, via the recording microelectrode, a passive bridge is used. Alternatively, drugs are added to the incubation bath.

EVALUATION

The resting membrane potential and paroxysmal depolarizations are recorded before and after application of drugs.

CRITICAL ASSESSMENT OF THE METHOD

The hippocampal slice has been one of the most useful models for the study of basic mechanisms underlying the epilepsies. The model has also been recommended for screening of putative anticonvulsant drugs.

MODIFICATIONS OF THE METHOD

Harrison and Simmonds (1985) performed quantitative studies on some antagonists of N-methyl-D-aspartate in slices of rat cerebral cortex consisting of cerebral cortex and corpus callosum.

Tissue culture models of epileptiform activity were described by Crain (1972).

Oh and Dichter (1994) studied the effect of a GABA uptake inhibitor on spontaneous postsynaptic currents in cultured rat hippocampal neurons by the whole cell patch clamp method.

Blanton et al. (1989) described whole cell recordings from neurons in slices of reptilian and mammalian cerebral cortex. Synaptic currents and membrane properties could be studied in voltage and current clamps in cells maintained within their endogenous synaptic currents.

Gähwiler (1988), Stoppini et al. (1991) described methods for organotypic cultures of nervous tissue. Hippocampal slices from 2–23-day old rats were maintained in culture at the interface between air and the culture medium. They were placed on a sterile, transparent and porous membrane and kept in Petri dishes in an incubator. This yielded thin slices which remained 1–4 layers thick and were characterized by a well preserved organotypic organization. Excitatory and inhibitory synaptic potentials could be analyzed using extra- or intracellular recording techniques. After a few days in culture, long-term potentiation of synaptic responses could reproducibly be induced.

Using this method, Liu et al. (1995) studied dopaminergic regulation of transcription factor expression in organotypic cultures of developing striatum of newborn rats.

Stuart et al. (1993) reported the implementation of infrared differential interference contrast video microscopy to an upright compound microscope and a procedure for making patch-pipette recordings from visually identified neuronal somata and dendrites in brain slices.

Bernard and Wheal (1995) described an *ex vivo* model of chronic epilepsy using slices of rat hippocampus previously lesioned by stereotactic injections of kainic acid. Extracellular population spikes were recorded from the stratum pyramidale of CA1 after stimulation by bipolar twisted wire electrodes placed in the stratum radiatum of CA1 area proximally to stratum pyramidale near the recording electrode.

Using hippocampal slices prepared from brain tissue of patients undergoing neurosurgery for epilepsy, Schlicker et al. (1996) showed that the serotoninergic neurons of the human hippocampus are endowed with presynaptic inhibitory autoreceptors.

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E.3.1.14 Electrical recordings isolated brain cells

PURPOSE AND RATIONALE

The use of the cell-attached patch clamp configuration to record action potential currents has shown to have utility in the testing for drug actions on ion channels in excitable cell membranes (Kay and Wong 1986; McLarnon 1990, 1991).

PROCEDURE

Preparation of cultured cells

The cultured cells are obtained from the hippocampus or the hypothalamus of rat brain. The isolation of the hippocampal CA1 neurones is performed according the procedure of Banker and Cowan (1977). The dissociated hypothalamic neurons are prepared according to Jirikowski et al. (1981). The hippocampal and hypothalamic neurons that are selected for electrophysiological recording are bipolar in shape with the long axis dimension between 10–15 µm. The neurons are studied over a period of 5–10 days after isolation.

Electrophysiology

The cell-attached patch clamp configuration is used to record spontaneous action potentials in the cultured neurons. The bath solution contains 140 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.3. The composition of the patch pipette solution is the same as the bath solution. The drugs used in the experiments are added to the bath solution.

The patch pipettes (Corning 7 052 glass) are fabricated using a specific patch pipette puller (PP-83; Narishige, Tokyo) are fire-polished and filled immediately prior to use. The resistance of the pipettes is in the range 4–8 MΩ and the tip diameters are between 1 and 2 µm. An axopatch amplifier (Axon Instruments, Foster City, CA), with low-pass filter set at 5 kHz, is used to record the capacitative currents. After recording, at a sampling frequency of 5 kHz, the data are stored on hard disc or video tape for subsequent analysis. All data are obtained at room temperature (21–24 °C).

EVALUATION

The capacitative component of current recorded by the patch pipettes is proportional to the rate of change of membrane potential and can be expressed as $I_C = C\,dV/dt$, where C is the specific membrane capacitance. Assuming a value of C of 1 µF/cm^2 and a tip diameter of the patch pipette of 2 µm, the membrane area isolated by the patch pipette is about 3×10^{-8} cm^2. Using a value of dV/dt of 100 mV/ms gives an approximate expected magnitude of I_C near 3 pA. When a class III antiarrhythmic drug that blocks a delayed rectifier K^+ channel is added to the bath, the portion of I_C corresponding to the after-hyperpolarization component of the action potential is completely abolished. The Na^+ spike is not altered by the drug. The cell-attached recordings of I_C can also be used to determine effects on the Na^+ spike when tetrodotoxin is included in the bath solution. Thus, the spontaneous action potential can be used for evaluation of drug effects on both K^+ and Na^+ channels in excitable membrane.

MODIFICATIONS OF THE METHOD

Chen et al. (1990) measured current responses mediated by $GABA_A$ receptors in pyramidal cells acutely dissociated from the hippocampus of mature guinea

pigs according to the procedure of Kay and Wong (1986) using whole-cell voltage-clamp recordings.

Caulfield and Brown (1992) studied inhibition of calcium current in NG108-15 neuroblastoma cells by cannabinoid receptor agonists using whole-cell voltage-clamp recordings.

Gola et al. (1992, 1993) performed voltage recordings on non-dissociated sympathetic neurones from rabbit coeliac ganglia using the whole-cell configuration of the patch clamp technique (Neher and Sakmann 1976; Sakmann and Neher 1983).

Stolc (1994) used the voltage-clamp technique in internally dialyzed single neurones isolated from young rat sensory ganglia to study the effects of pyridoindole stobadine on inward sodium and calcium currents and on slow non-inactivating components of potassium outward current.

McGivern et al. (1995) examined the actions of a neuroprotective agent on voltage dependent Na^+ currents in the neuroblastoma cell line, NIE-115, using the whole cell variant of the patch clamp technique.

Smith (1995) reviewed the use of patch- and voltage clamp procedures to study neurotransmitter transduction mechanisms.

Using whole-cell and perforated-patch recordings, Delmas et al. (1998) examined the part played by endogenous G-protein $\beta\gamma$ subunits in neurotransmitter-mediated inhibition of N-type Ca^{2+} channel current in dissociated rat superior cervical sympathetic neurones.

Gonzales et al. (1985) registered membrane potentials with intracellular electrodes in cultured olfactory chemoreceptor cells.

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E.3.1.15 Isolated neonatal rat spinal cord

PURPOSE AND RATIONALE

The spinal cord of the neonatal rat is a useful *in vitro* preparation, originally proposed by Otsuka and Konishi (1974). In this preparation, ventral root potentials of ten seconds of duration can be recorded after supramaximal electrical stimulation of the lumbar dorsal root. Variously implicated in the generation of these slow ventral root potentials are tachykinins, such as substance P and neurokinin B (Yanagisawa et al. 1982; Akagi et al. 1985; Otsuka and Yanagisawa 1988; Guo et al. 1998) and agonists at the glutamate receptor sites (Evans et al. 1982; Ohino and Warnick 1988 1990; Shinozaki et al. 1989; Ishida et al. 1990, 1991, 1993; Woodley and Kendig 1991; Bleakman et al. 1992; King et al. 1992; Thompson et al. 1992; Zeman and Lodge 1992; Pook et al. 1993; Jane et al. 1994; Boxall et al. 1996). These long lasting reflexes are thought to reflect a nociceptive reflex for several reasons; the threshold of activation corresponds to that of C fibre primary afferents (Akagi et al. 1985); they can be depressed by opioids (Yanagisawa et al. 1985; Nussbaumer et al. 1989; Faber et al. 1997) and α_2-adrenoceptor agonists (Kendig et al. 1991) and a similar response can be evoked by peripheral noxious stimulation (Yanagisawa et al. 1995).

PROCEDURE

Preparation of spinal cord

Male Wistar rats aged 6–9 days are used. Under ether anesthesia the spinal column is quickly removed from the animal and placed in a Petri dish, filled with oxygenated physiological solution. A laminectomy is performed on the dorsal surface of the spinal column at room temperature. The spinal cord of the mid-thoracic to mid-sacral level is then carefully removed from the column and hemisected in the longitudinal plane under a dissecting microscope. After removal of the dura mater, the hemisected cord is completely submerged in the recording chamber (total volume: approximately 0.5 ml), which is perfused with physiological solution (124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 15 mM $NaHCO_3$, 11 mM glucose) at a flow rate of 1.5 to 2.5 ml/min. The perfusion medium is continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 and the temperature is kept at 25 ±0.5 °C. The cut ends of the corresponding dorsal and ventral roots in an $L_{3\text{-}5}$ segment are fixed to a pair of suction electrodes for stimulating and recording. The preparation is stabilized in the recording chamber for at least 90 min to allow recovery from the dissection and the sealing of the roots to suction electrodes.

Recording of monosynaptic reflexes

Test stimulations, composed of square wave pulses of 0.05–0.2 ms duration and 5–30 V, are applied to the dorsal root every 10 s. The discharges of the corresponding ventral root are recorded with a suction electrode, amplified and monitored on an oscilloscope and stored on an analog data recorder or computer discs for later analysis. The mean values for the waveform of the monosynaptic reflex (amplitude, area and latency) are obtained from 6–18 successive responses in each experiment before and during application of drugs.

Recording of single motoneuron activity

Test pulses (0.01–0.1 ms duration and 5–15 V) are applied to the dorsal or ventral root every 2 s. The activity of single motoneurons is recorded extracellularly using glass microelectrodes (electrical resistance approximately 10–30 MΩ) filled with 3 M sodium chloride or 2 M sodium acetate. The microelectrode is inserted into the ventral part of the cord through the hemisected surface while monitoring the field potential. The motoneurons in the ventral horn are identified by the short and consistent latency of antidromic spikes (1.66 ±0.46 ms, $n = 5$), following the stimulation of the ventral root. The motoneurons also produce transsynaptic spikes with orthodromic stimulation of the dorsal root, of which the latency is 10.26 ±1.05 ms upon supramaximal stimulation. The spike generation of motoneurons is displayed on an oscilloscope and stored on magnetic tapes. The spontaneous firing of the motoneuron is also monitored on an oscilloscope and recorded through a window discriminator and spike counter. The mean number and latency of spikes and latency of the dorsal root-elicited spikes are obtained from 20–40 successive responses in each experiment. Comparisons are made before and 3–5 min after application of drugs.

EVALUATION

All data are expressed as the mean ±SEM. Statistical significance of the data is determined by repeated measures analysis of variance (ANOVA) and, when appropriate, Student's *t*-test. A *P* value of less than 0.05 is considered statistically significant.

MODIFICATIONS OF THE METHOD

Smith and Feldman (1987), Wong et al. (1996) described an *in vitro* neonatal rat brainstem/spinal cord preparation. The brainstem and cervical spinal cord were isolated from 0–4 days old ether anesthetized Sprague Dawley rats. The en bloc neuraxis was pinned down with ventral surface upward in a recording chamber and superfused continuously with artificial cerebrospinal fluid. Respiratory activity was recorded with suction electrodes from the C_4 ventral root.

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E.3.1.16 Cell culture of neurons

PURPOSE AND RATIONALE

Cell culture of neurons, especially of hippocampal neurons, has become a widely used tool in pharmacological studies (Banker and Cowan 1977; Skaper et al. 1990, 1993, 2001; Araujo and Cotman 1993; Brewer 1997, 1999; Brewer et al. 1998; Li et al. 1998; Mitoma et al. 1998; Semkowa et al. 1998, 1999; Chaudieu and Privat 1999; May et al. 1999; Hampson et al. 2000; Novitskaya et al. 2000; Pickard et al. 2000; Vergun et al. 2001).

The basic information on methodology of cell culture of rat hippocampal neurones was given by Banker and Cowan (1977). One modification used by Skaper et al. (1990, 2001) studying the role of mast cells on potentiation by histamine of synaptically mediated excitotoxicity in cultured hippocampal neurones is described below.

PROCEDURE

Preparation of hippocampus

Timed pregnancies are obtained in female Sprague Dawley rats by daily checking vaginal washings for sperm, the day on which sperm is found being regarded as day 0. At the appropriate stage of gestation the pregnant rats are anesthetized and the uterus removed to a sterile dish. The remainder of the cell preparations is performed in a sterile hood.

The brains are removed from the fetuses with a pair of fine scissors, and the cerebral hemispheres separated from the brain stem. When the hemisphere of an 18- to 19-day-old fetus in viewed in a dissecting microscope the hippocampus can be clearly seen on its medial surface. The hippocampal fissure, usually marked by a conspicuous group of blood vessels, indicates the approximate junction between the hippocampus and the adjoining subicular and entorhinal cortex. The developing fimbria is seen as a white translucent band along the free margin of the hippocampus. Before separating the hippocampus from the hemisphere,

the meninges and adherent chorioid plexus are carefully pulled off with fine forceps. At this stage the full depth of the hippocampal fissure can be seen. Then with iridectomy scissors the hippocampus is separated from the adjoining cortex by a cut parallel to the hippocampal fissure, and by transverse cuts at its rostral and caudal ends.

Cell culture

Hippocampi isolated from embryonic rats (gestational age 17.5 days) are incubated with 0.08% trypsin, and dissociated in Neurobasal medium containing 10% heat-inactivated calf serum. Cells are pelleted by centrifugation (200 *g*, 5 min) and resuspended in Neurobasal medium containing B27 (Life Technologies, Inc.) supplements (with antioxidants), 25 µM glutamate, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. The cell suspension is plated onto poly-D-lysine (10 µg/ml) coated 48-well culture plates at a density of 4.5×10^4 cells per cm^2. Cultures are maintained at 37 °C in a humidified atmosphere of 5% CO_2-95% air. After 5 days, one-half of the medium is replaced with an equal volume of maintenance medium (plating medium but containing B27 supplements without antioxidants, and lacking glutamate). Additional medium exchanges (0.5 volume) are performed every 3–4 days thereafter. Cells are used between 14 and 16 days in culture. During this period, neurones develop extensive neuritic networks, and form functional synapses.

Mast cells are collected from the peritoneal lavage of male Sprague Dawley rats and isolated over a bovine serum albumin gradient to >90% purity, as judged by toluidine blue and safranin staining.

Neurotoxicity assays

Cultures are washed once with Locke's solution (pH 7.0–7.4) with or without 1 mM $MgCl_2$. Drug treatments are carried out for 15–30 min (25 °C) in a final volume of 0.5 ml. In the case of mast-cell-neurone cocultures, transwell inserts (3-µm pore size, 9 mm diameter) are seeded with 5×10^4 mast cells in RPMI-1 640 medium and placed in 24-well plates overnight. Inserts with mast cells are then placed into wells with hippocampal cells. Mast cells activation is achieved using an antigenic stimulus (0.3 µg/ml anti-DNP IgE/0.1 µg/ml DNP albumin). The mast cell containing inserts are removed at the end of the Mg^{2+}-free incubation. After this time all cell monolayers are washed with complete Locke's solution and returned to their original culture medium for 24 h. Cytotoxicity is evident during 24 h after the insult. Viable neurons have phase-bright somata of round-to-oval shape, with smooth, intact neurites. Neurones are considered nonviable when they exhibit neurite fragmentation and somatic swelling and vacuolation. Cell survival is quantified 24 h after the insult by a colorimetric reaction with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

EVALUATION

Data are analyzed by one-way ANOVA with Student-Newman-Keuls *post hoc* test for differences between groups.

MODIFICATIONS OF THE METHOD

Brewer (1997) reported the isolation and culture of adult rat hippocampal neurons. Using different proteases and special separation techniques, about 90 000 viable neurons could be isolated from each hypothalamus at any age rat from birth to 24–36 months. Neurons were cultured for more than 3 weeks.

Flavin and Ho (1999) found that propentofylline protects hippocampal neurons in culture from death triggered by macrophage or microglia secretory products.

To study neurite outgrowth in cultured hippocampal cells from Wistar rat embryos, 5 000 cells/well were seeded in 8-well LabTec tissue culture slides with a grown surface of permanox plastic and grown in Neuro-basal medium supplemented with B27 (Life Technologies, Inc.), 20 mM HEPES, 0.4% bovine serum albumin, penicillin (100 IU/ml), and streptomycin (100 µg/ml). (Novitskaya et al. 2000). For image analysis, cells were fixed in 4% paraformaldehyde and stained for 20 min with Coomassie Blue R250. Cover slides were observed in an inverted microscope using phase contrast optics. To measure neurite outgrowth from hippocampal neurons, an unbiased counting frame containing a grid with a number of test lines was superimposed on the images of cells. The number of intersections of cellular processes with the test lines was counted and related to the number of cell bodies, thereby allowing quantification of neurite length per cell.

Cell culture experiments were also performed with **neuronal cells from other areas of the brain** besides the hippocampus.

Brain tissue samples of rat embryos containing either septum plus preoptic area or retrochiasmatic hypothalamus were dissociated and cultured for 14 and 21 days by Jirikowski et al. (1981). By means of immunofluorescence, LHRH, α-MSH, vasopressin and neurophysin-containing hormones could be identified.

Sinor et al. (2000) studied NMDA and glutamate evoked excitotoxicity at distinct cellular locations in rat cortical neurones *in vitro*.

Canals et al. (2001) examined neurotrophic and neurotoxic effects of nitric oxide on neuronal enriched fetal midbrain cultures from embryonic Sprague Dawley rats.

López et al. (2001) investigated the release of amino acid neurotransmitters in cultured cortical neurons obtained from gestation day 19 rats by nicotine stimulation.

Ehret et al. (2001) studied the modulation of electrically evoked acetylcholine release in cultured septal neurones from embryonic Wistar rats.

Tang et al. (2001) found a lack of replicative senescence in cultured rat oligodendrocyte precursor cells.

Yamagishi et al. (2001) used cultured rat cerebellar granule neurons as a model system for studying neuronal apoptosis.

Noh and Koh (2000) prepared mixed **mouse** cortical cultures containing both neurons and astrocytes, and pure astrocyte cultures, from fetal (15 d of gestation) and neonatal (1–3 postnatal days) mice.

Ushida et al. (2000) succeeded to directly isolate clonogenic **human** central nervous system stem cells from fresh human brain tissue, using antibodies to cell surface markers and fluorescence-activated cell sorting.

For further studies with brain cell cultures see Sect. F.2.0.10.

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E.3.2 *In vivo* methods

E.3.2.1 Electroshock in mice

PURPOSE AND RATIONALE

The electroshock assay in mice is used primarily as an indication for compounds which are effective in grand mal epilepsy. Tonic hindlimb extensions are evoked by electric stimuli which are suppressed by anti-epileptics but also by other centrally active drugs.

PROCEDURE

Groups of 6–10 male NMRI mice (18–30 g) are used. The test is started 30 min after i.p. injection or 60 min after oral treatment with the test compound or the vehicle. An apparatus with corneal or ear electrodes (Woodbury and Davenport 1952) is used to deliver the stimuli. The intensity of stimulus is dependent on the apparatus, e.g. 12 mA, 50 Hz for 0.2 s have been used. Under these conditions all vehicle treated mice show the characteristic extensor tonus.

EVALUATION

The animals are observed closely for 2 min. Disappearance of the hindleg extensor tonic convulsion is used as positive criterion. Percent of inhibition of seizures relative to controls is calculated. Using various doses, ED_{50}-values and 95% confidence interval are calculated by probit analysis.

ED_{50}-values after oral administration are:

- Diazepam 3,0 mg/kg
- Diphenylhydantoin 20,0 mg/kg

CRITICAL ASSESSMENT OF THE METHOD

The electro-shock test in mice has been proven to be a useful tool to detect compounds with anticonvulsant activity.

MODIFICATIONS OF THE METHOD

Cashin and Jackson (1962) described a simple apparatus for assessing anticonvulsant drugs by the electroshock seizure test in mice.

Kitano et al. (1996) developed the increasing-current electroshock seizure test, a new method for assessment of anti- and pro-convulsant activities of drugs in mice. A single train of pulses (square wave, 5 ms, 20 Hz) of linearly increasing intensity from 5 to 30 mA was applied via ear electrodes. The current at which tonic hindlimb extension occurred was recorded as the seizure threshold. The method allows the determination of seizure threshold current for individual animals.

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E.3.2.2 Pentylenetetrazol test in mice and rats

See Sect. E.2.2.1.

E.3.2.3 Strychnine-induced convulsions in mice

See Sect. E.2.2.2.

E.3.2.4 Picrotoxin-induced convulsions in mice

See Sect. E.2.2.3.

E.3.2.5 Isoniazid-induced convulsions in mice

See Sect. E.2.2.4.

These tests, already described for evaluation of the anticonvulsive activity of anxiolytics, can be used and show activity for anti-epileptics.

Many other agents induce seizures in animals and have been used to test the anticonvulsant activity of drugs (Stone 1972), e.g. glutarimides (Hahn and Oberdorf 1960), pilocarpine (Tursky et al. 1987), methionine sulfoximine (Toussi et al. 1987), *N*-methyl-D-aspartic acid (Leander et al. (1988), γ-hydroxybutyrate (Snead 1988).

Shouse et al. (1989) described mechanisms of seizure suppression during rapid-eye-movement (REM) sleep in cats. Spike-wave paroxysms in the EEG accompanied by bilateral myoclonus of the head and the neck were induced by i.m. injection of 300 000 to 400 000 IU/kg sodium penicillin G.

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E.3.2.6 Bicuculline test in rats

PURPOSE AND RATIONALE

Seizures can be induced by the $GAGA_A$-antagonist bicuculline and are antagonized by known anti-epileptics.

PROCEDURE

Female Sprague-Dawley rats are injected i.v. with 1 mg/kg bicuculline. At this dose, a tonic convulsion appears in all treated rats within 30 s after injection. Test compounds are administered orally 1 or 2 h before bicuculline injection. Dose-response curves can be obtained.

EVALUATION

Percentage of protected animals is evaluated. ED_{50}-values and 95% confidence limits are calculated by probit analysis.

CRITICAL ASSESSMENT OF THE METHOD

Like the electroshock test, the bicuculline test is considered to be relatively specific for anti-epileptic activity.

MODIFICATIONS OF THE METHOD

Czuczwar et al. (1985) studied the antagonism of N-methyl-D,L-aspartic acid-induced convulsions by antiepileptic drugs and other agents.

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E.3.2.7 4-aminopyridine-induced seizures in mice

PURPOSE AND RATIONALE

The K^+ channel antagonist 4-aminopyridine is a powerful convulsant in animals and in man. The drug read-

ily penetrates the blood-brain barrier and is believed to induce seizure activity by enhancing spontaneous and evoked neurotransmitter release. Although both excitatory and inhibitory synaptic transmission is facilitated by 4-aminopyridine, the epileptiform activity induced by the drug is predominantly mediated by non-NMDA type excitatory amino acid receptors. In mice, parenterally administered 4-aminopyridine induces clonic-tonic convulsions and lethality.

PROCEDURE

Male NIH Swiss mice weighing 25–30 g are allowed to acclimatize with free access to food and water for a 24-h period before testing. Test drugs are administered in various doses intraperitoneally 15 min prior to s.c. injection of 4-aminopyridine at a dose of 13.3 mg/kg which was found to be the LD_{97} in this strain of mice. Controls treated with 4-aminopyridine only exhibit characteristic behavioral signs, such as hyperreactivity, trembling, intermitted forelimb/hindlimb clonus followed by hindlimb extension, tonic seizures, opisthotonus and death. The mean latency to death at the LD_{97} is about 10 min. Groups of 8 mice are used for each dose.

EVALUATION

The percentage of protected animals at each dose is used to calculate ED_{50} values. Phenytoin-like anti-convulsants such as carbamazepine and broad spectrum anticonvulsants such as phenobarbital and valproate are effective whereas GABA-enhancers such as diazepam, several NMDA antagonists, and CA^{2+} channel antagonists such as nimodipine are not.

CRITICAL ASSESSMENT OF THE METHOD

The profile of drugs effective in this seizure model is distinct from other chemoconvulsant models and more similar to those that prevent tonic hindlimb extension in the maximal electroshock seizure test. The test is useful to differentiate the mode of action of anticonvulsant drugs.

MODIFICATIONS OF THE METHOD

Morales-Villagran et al. (1996) described protection against seizures induced by intracerebral or intra-cerebroventricular administration of 4-aminopyridine by NMDA receptor antagonists.

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E.3.2.8 Epilepsy induced by focal lesions

PURPOSE AND RATIONALE

Intrahippocampal injections of noxious agents or certain cerebral lesions can induce seizures in animals. Cavalheiro et al. (1982) studied the long-term effects of intrahippocampal kainic acid injections in rats.

PROCEDURE

Adult male Wistar rats are anesthetized with a chloral hydrate/Nembutal® mixture and placed in a stereotactic apparatus. For injections, a 0.3 mm cannula is inserted through a burr hole in the calvarium. The coordinates for hippocampal injections are based on a stereotactic atlas, e.g., Albe-Fessard et al. (1971). Kainic acid is dissolved in artificial serum and infused in various doses (0.1 to 3.0 µg) in a volume of 0.2 µl over a period of 3 min. For recording, bipolar twisted electrodes (100 µm) are positioned stereotaxically and fixed on the skull with dental acrylic cement. Depth recording sites include the dorsal hippocampus and amygdala ipsilateral to the injected side. Surface electrodes are guided from jeweler's screws over the occipital cortex. An additional screw in the frontal sinus serves as indifferent electrode for grounding. Signals are recorded by an EEG polygraph.

EVALUATION

EEG recordings and observations of convulsive seizures are performed during the acute phase and during the chronic phase (up to 2 months) with and without drug treatment.

MODIFICATIONS OF THE METHOD

Several agents have been used as convulsants after topical administration, e.g., application of alumina cream (Kopeloff et al. 1942, 1955; Ward 1972; Feria-Velasco et al. 1980), implantation of cobalt powder (Dow et al. 1962; Fischer et al. 1967), injection of a colloidal gel of tungstic acid (Blum and Liban 1960; Black et al. 1967), topical application of penicillin (Matsumoto and Marsan 1964), subpial injection of saturated $FeCl_3$ solution (Reid et al. 1979; Lange et al. 1980), intracer-

ebral injections of zinc sulfate (Pei et al. 1983), intracerebral injection of antibodies to brain gangliosides (Karpiak et al. 1976, 1981), microinjections of cholinergic agonists (Ferguson and Jasper 1971; Turski et al. 1983), topical application of atropine (Daniels and Spehlman 1973), injection of tetanus toxin into the hippocampus (Mellanby et al. 1984; Hawkins and Mellanby 1987), injection of strychnine in the visual or somatosensory cortex (Atsev and Yosiphov 1969), electrophoretic application of bicuculline from a fluid filled microelectrode (Campell and Holmes 1984).

Bernhard et al. (1955, 1956) evaluated the anticonvulsive effect of local anaesthetics in cats and monkeys. The head was fixed in light Nembutal anaesthesia, the parietal areas exposed and covered with paraffin oil. Stimulating electrodes were placed at the surface of the parietal region. The cortex was stimulated with repetitive square wave shocks (duration 1–3 ms) with a frequency of 25 per s for 5 s. In order to avoid muscular movements, d-tubocurarine was given. Cortical afterdischarge was registered before and after injection of local anaesthetics.

Cortical epileptic lesions were produced by local freezing (Stalmaster and Hanna 1972; Hanna and Stalmaster 1973; Loiseau et al. 1987).

Repetitive electrical stimulation of discrete regions of the central nervous system has been used as a convenient method for reproduction of the ictal phenomena of epilepsy (Marsan 1972; Racine 1972).

Remler and Marcussen (1986), Remler et al. (1986) studied the pharmacological response of systemically derived focal epileptic lesions. A defined area of left hemisphere of rats was radiated by α-particles from a cyclotron destroying the blood brain barrier. After a period of 150 days following irradiation, bicuculline was injected intraperitoneally resulting in focal lesions with EEG spikes and convulsions. Anticonvulsant drugs decreased these effects.

Walton and Treiman (1989) and Walton et al. (1994) described a model of cobalt-lesioned rats in which status epilepticus was induced by injection of homocysteine thiolactone.

Anderer et al. (1993) pointed out that restriction to a limited set of EEG-target variables may lead to misinterpretation of pharmaco-EEG results.

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E.3.2.9 Kindled rat seizure model

PURPOSE AND RATIONALE

Kindling, first described by Goddard et al. (1969), results from repetitive subconvulsive electrical stimulation of certain areas of the brain. Initially, local afterdischarge is associated with mild behavioral signs; however, with continued stimulation electrical activity presumably spreads, and generalized convulsions occur. Although the pathogenesis of kindled seizures is not fully understood, it serves as a useful tool for investigating the efficacy of experimental anticonvulsant agents.

PROCEDURE

Adult female Sprague-Dawley rats (270–400 g) are used. The rats are implanted with an electrode in the right amygdala according to the coordinates of Pellegrino et al. (1979): frontal, 7.0; lateral, –4.7; horizontal, 2.5. At least 1 week has to elapse before electrical stimulation of the brain is started. After discharge threshold is determined for each rat. Duration and amplitude, behavioral seizure duration and seizure stage are recorded with increased stimuli afterdischarges. Seizure severity is classified into 5 stages (Racine 1972). Rats are considered to be kindled on the first stimulation causing a stage 5 seizure which is followed by at least 2 consecutive stage 5 seizures.

The animals are tested on the day before and after treatment with the test compound (i.p. or orally). Amygdala stimulation is applied at various time intervals.

EVALUATION

The occurrence and the degree of seizures are compared between control results and the those after administration of the test compound.

CRITICAL ASSESSMENT OF THE METHOD

The kindled seizure model offers an approach to study anticonvulsive drugs on the basis of a pathophysiological model. This method may give more relevant results than the simpler methods using maximal electroshock or chemically induced convulsions.

MODIFICATIONS OF THE METHOD

Generalized convulsive seizures have been induced by daily amygdaloid stimulation in **baboons** (Wada and Osawa 1976), and in **rhesus monkeys** (Wada et al. 1978).

The kindling effect can be produced by intermittent administration of small doses of pentylenetetrazol (Mason and Cooper 1972).

Dürmüller et al. (1994) tested a competitive (NBQX) and a non-competitive (GYKI 52 446) AMPA antagonist, and a competitive NMDA antagonist (D-CPPene) against the development of kindling and against fully kindled seizures in amygdala-kindled rats.

Croucher et al. (1996) described a chemical kindling procedure in rats by daily focal microinjection of NMDA into the right basolateral amygdala and the inhibition of seizures by a NMDA receptor antagonist.

Suzuki et al. (1996) studied the anticonvulsant action of metabotropic glutamate receptor agonists in kindled amygdala of rats.

The kindling procedure can also be used to evaluate antidepressant drugs (Babington 1975).

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E.3.2.10
Posthypoxic myoclonus in rats

PURPOSE AND RATIONALE

The syndrome of posthypoxic myoclonus in man was described by Lance and Adams (1963). Lance (1968), Fahn (1986). Troung et al. (1994), Jaw et al. (1994, 1995, 1996) reported on a model in rats resembling this human disorder.

PROCEDURE

Male Sprague Dawley rats fasted 12–24 h prior surgery are anesthetized with 100 mg/kg ketamine i.p., supplemented by 0.4 mg/kg atropine. The animal is placed on a circulating water pad and kept at a constant body temperature by a heating lamp. The rat is intubated and ventilated with 30% O_2 in N_2O. A femoral artery and vein are cannulated for monitoring blood pressure and delivery of drugs, respectively. Electrocardiogram and blood pressure are recorded with a polygraph. The rat is then paralyzed with 2 mg/kg succinlycholine i.v. and ventilator settings are adjusted to a rate of 60 strokes/min and a volume of 7.5 ml/kg, which yields blood gases of >150 mm Hg pO_2, 35–40 mm Hg pCO_2, and a pH of 7.35–7.40. N_2O is replaced with N_2 and an equilibrium period of 5 min is allowed.

Cardiac arrest is accomplished with a trans-thoracic intracardiac injection of KCl and cessation of the respiration. Resuscitation is begun 10 min after the arrest by turning on the ventilator (100% O_2), manual thoracic compressions, and i.v. injections of 20 µg/kg epinephrine hydrochloride and sodium bicarbonate (4 mEq/kg). The rat is then weaned from the ventilator over 2–4 h and extubated.

Auditory-induced myoclonus: Rats are presented with a series of 45 clicks from a metronome (1 Hz, 95 dB, 40 ms), and the response to each click is scored as follows: 0 = no response, 1 = ear twitch, 2 = ear and head jerk, 3 = ear, head, and shoulder jerk, 4 = whole body jerk, 6 = whole body jerk of such severity that it causes a jump. The total myoclonus score of each rat is determined by summing up the scores yielded over 45 clicks.

Since rats ranging from 3 to 14 days post cardiac arrest show similar susceptibility to audiogenic stimulation, animals within this period are used for pharmacological tests. Myoclonus scores are assessed 30 min before and 60 min after intraperitoneal drug application.

EVALUATION

Changes in myoclonus scores are analyzed by paired two-tailed Student's *t*-test.

CRITICAL ASSESSMENT OF THE TEST

Some anticonvulsant drugs, such as clonazepam and valproic acid were reported to be active in this test; however, phenytoin is not. Posthypoxic myoclonus may present a special pathological condition.

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E.3.2.11 Genetic animal models of epilepsy

PURPOSE AND RATIONALE

Several animal species exhibit epilepsy with spontaneous recurrent seizures such as dogs, rats, and mice (Löscher 1984). Serikawa and Yamada (1986) described spontaneous epileptic rats which are double mutants and exhibit both tonic and absence-like seizures.

PROCEDURE

Spontaneous epileptic rats are obtained by mating the tremor heterozygous rat (*tm/+*) with the zitter homozygous rat (*zi/zi*) found in a Sprague-Dawley colony. The behavior of the spontaneous epileptic rats is recorded weekly for 2 h on videotapes. The frequency of tonic convulsions and wild jumping occurring in the absence of external stimuli are recorded. Under anesthesia silver ball-tipped and monopolar stainless-steel electrodes are chronically implanted in the left frontal cortex and hippocampus. An indifferent electrode is placed on the frontal cranium. The frequency of absence-like seizures and tonic convulsions, as well as the duration of each seizure, are measured on the EEG. A mild tactile stimulus is given on the back of the animal every 2.5 min to induce consistent tonic convulsions. Compounds are given i.p. or orally.

EVALUATION

The number of seizures and the duration of each seizure are obtained and the total duration of the seizures (number × duration) is calculated every 5 min before and after injection of the drug. Percent changes between values before and after drug administration are calculated.

CRITICAL ASSESSMENT OF THE TEST

Studies in spontaneous epileptic rats and other genetic models are of value for an in depth investigation of a potential anti-epileptic drug.

MODIFICATIONS OF THE METHOD

The **genetically epilepsy-prone rat GEPR-9** has been described by Faingold et al. (1992, 1994) and by Jobe et al. (1992). The inferior colliculus is strongly implicated as a critical initiation site within the neuronal network for audiogenic seizures.

Several other genetic animal models have been described (Löscher and Frey 1984; Löscher and Meldrum 1984) showing epilepsy with spontaneous recurrent seizures, such as

dogs (Cunningham 1971; Edmonds et al. 1979),

rats with petit mal epilepsy (Vergnes et al. 1982), rats with two mutations, zitter and tremor (Serikawa and Yamada 1986; Xie et al. 1990), rats with absence-like states and spontaneous tonic convulsions (Sasa et al. 1988),

tottering mice (Green and Sidman 1962; Noebels 1979; Noebels and Sidman 1979; Fletcher et al. 1966),

leaner mutant mice with severe ataxia and atrophic cerebellum (Herrup and Wilczynnski 1982; Heckroth and Abbott 1994),

the **quaking mouse** (Sidman et al. 1966; Chermat et al. 1981) having deficiencies in myelinization in the nervous system (Hogan 1977; Li et al. 1993; Bartoszewicz et al. 1995) and alterations in the dopaminergic (Nikulina et al. 1995) and α_2-adrenergic (Mitrovic et al. 1992) brain system,

epilepsy with reflex seizures, such as

baboons with photomyoclonic seizures (Killam et al. 1966, 1967; Stark et al. 1970; Naquet and Meldrum 1972; Smith et al. 1991; Chapman et al. 1995),

photosensitive **fowls** (Crawford 1969, 1970),

audiogenic seizure susceptible mice (Collins 1972; Seyfried 1979; Chapman et al. 1984; Stenger et al. 1991),

mechanically stimulated mice (Imaizumi et al. 1959; Oguro et al. 1991),

audiogenic seizure susceptible rats (Consroe et al. 1979; Reigel et al. 1986; Smith et al. 1991; Patel et al. 1990),

gerbils with reflex seizures (Thiessen et al. 1968; Loskota et al. 1974; Majkowski and Kaplan 1983; Bartoszyk and Hamer 1987).

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E.4 Hypnotic activity

E.4.0.1 General considerations

The term "hypnotic" has to be defined. In man, the purpose of taking hypnotics is to obtain a "normal" night's sleep from which the patient can be aroused without any subsequent hangover. In animal experiments, the term "hypnotic" has been applied to a much deeper stage of central depression of drug induced unconsciousness associated with loss of muscle tone and of righting reflexes. Therefore, most of the pharmacological models are questionable in regard to their predictivity to find an ideal hypnotic for human therapy. Many of the pharmacological tests are based on the potentiation of sleeping time induced by barbiturates or other sedative agents.

Since the biochemical events during sleep are rather unknown no *in vitro* method exists for testing compounds with potential hypnotic activity.

E.4.1 *In vivo* methods

E.4.1.1 Potentiation of hexobarbital sleeping time

PURPOSE AND RATIONALE

The test is used to elucidate CNS-active properties of drugs. Not only hypnotics, sedatives, and tranquilizers but also antidepressants at high doses are known to prolong hexobarbital induced sleep after a single dose of hexobarbital. The loss of righting reflex is measured as criterion for the duration of hexobarbital-induced sleeping time. Mice are used in this test, since metabolic elimination of hexobarbital is rapid in this species.

PROCEDURE

Groups of 10 male NMRI-mice with an average weight of 18–22 g are used. They are dosed orally, i.p. or s.c. with the test compound or the reference standard (e.g. 3 mg/kg diazepam p.o.) or the vehicle. Thirty min after i.p. or s.c. injection or 60 min after oral dosing 60 mg/kg hexobarbital is injected intravenously. The animals are placed on their backs on a warmed (37 °C) pad and the duration of loss of the righting reflex (starting at the time of hexobarbital injection) is measured until they regain their righting reflexes. Injection of 60 mg/kg hexobarbital usually causes anesthesia for

about 15 min. If there is any doubt as to the reappearance of the righting reflex, the subject is placed gently on its back again and, if it rights itself within one minute, this time is considered as the endpoint.

EVALUATION

Mean values of duration of anesthesia (min) are recorded in control and experimental groups. The percent change in duration of anesthesia is calculated in the experimental groups as compared to those of the controls. ED_{50} values can be calculated. ED_{50} is defined as the dose of drug leading to a 100% prolongation in duration of anesthesia in 50% of the animals.

CRITICAL ASSESSMENT OF THE METHOD

The anxiolytic agents of the benzodiazepine type show an uniform pattern with oral ED_{50} values of less than 1 mg/kg. This is in agreement with the fact that barbiturates also show anxiolytic activity in anti-anxiety tests with animals as well as in patients. Neuroleptics, such as chlorpromazine and haloperidol, also prolong hexobarbital sleeping time in low doses. The test is considered to be unspecific since compounds which inhibit liver metabolism of hexobarbital also prolong time of anesthesia. Balazs and Grice (1963) discussed the relationship between liver necrosis, induced by CCl_4 or nitrosamines, and pentobarbital sleeping time in rats.

OTHER USES OF THE TEST

Hexobarbital sleeping time is not only prolonged by the simultaneous administration of many compounds but also shortened under special conditions. Several CNS-active compounds (analeptics and stimulants like amphetamine and related compounds and methylxanthines) reduce hexobarbital sleeping time. Standard compounds for this kind of procedure are pentylenetetrazol, methamphetamine and aminophylline.

After repeated administrations, induction of metabolic enzymes in the liver is caused by many compounds and leads to an increased destruction of hexobarbital. Due to the accelerated metabolism of hexobarbital, sleeping time is reduced.

MODIFICATIONS OF THE TEST

Instead of hexobarbital, another barbiturate, thiopental can be used which has been proven in clinical use to be a short acting anesthetic. Test compounds or the standard are given 60 min before i.v. injection of 25 mg/kg thiopental to mice with a weight between 18 and 22 g. The animals are placed on their backs and the re-appearance of the righting reflex is observed. The ED_{50} which results in a 100% prolongation in duration of anesthesia is between 2.5 and 4.0 mg/kg diazepam p.o.

Simon et al. (1982) tested the interaction of various psychotropic agents with sleep induced by barbital or pentobarbital in mice. Pentobarbital (50 mg/kg) or barbital (180 mg/kg) were injected i.p. and the latency and duration of sleep (loss of righting reflex) were recorded. The test compound was usually administered i.p. 30 min before the injection of the barbiturate. The test was recommended for detecting sedative or anti-sleep activity. Since pentobarbital is metabolized by the liver whereas barbital is not, a comparative study using the two compounds can be useful for determining whether an eventual potentiation or antagonism can be ascribed to enzymatic inhibition or induction.

Fujimori (1965) recommended the use of barbital-Na instead of hexobarbital for the sleeping time test since barbital is not biotransformed by the liver microsomal system.

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E.4.1.2 Experimental insomnia in rats

PURPOSE AND RATIONALE

James and Piper (1978) described a method for evaluating potential hypnotic compounds in rats. Usually, the compounds are tested in normal animals where they do not significantly decrease wakefulness. Footshock induced "insomnia" in rats is proposed as suitable model for insomnia in patients.

PROCEDURE

Male Wistar rats (200–275 g) are prepared for chronic electroencephalographic and electromyographic recordings. Four silver/silver chloride epidural electrodes and two disc nuchal electrodes are implanted. A mini-

mum of 10 days is allowed for recovery from surgery. The animals are placed into sound-attenuated recording chambers with grid floors. The frontal-occipital electroencephalogram and the electromyogram are recorded via nonrestraining recording leads on a polygraph and a tape recorder.

On the control day, the animals are dosed with the vehicle and a control nonstress recording is obtained for 8 h. On the next day, the animals are again injected with the vehicle and then exposed to electric footshocks for 8 h. The footshock is delivered through the grid floor of the recording chamber using the EMG leads as indifferent electrodes, in the form of a 0.5 mA pulse of 15 ms width for 30 s at 1 Hz. During the footshock the EEG and EMG recording circuits are automatically interrupted. The delivery of electric footshock is triggered automatically by two adjustable timers. In this way, each shock period of 30 s is followed by an interval of 30 min. On the next day the rats are dosed with the test compound or the standard and recordings are obtained during a shock session of 8 h.

EVALUATION

The sleep-wake cycle is definitely altered by the stress procedure. The amounts of arousal and of slow wave sleep I are increased, whereas slow wave sleep II and paradoxical sleep are decreased. Phenobarbital and benzodiazepines antagonize these changes at least partially.

CRITICAL ASSESSMENT OF THE METHOD

For screening procedures, the method is too expensive and time-consuming. However, the EEG-parameters in a situation of insomnia similar to men can indicate the usefulness of a new compound.

MODIFICATIONS OF THE METHOD

Gardner and James (1987) described a modified shortened protocol in which a 2.5-h nonstressed control period is followed by drug or vehicle administration and a further 5.5-h recording of the electrocorticogram in the presence of intermittent footshock.

Laval et al. (1991) studied the effect of anxiolytic and hypnotic drugs on sleep circadian rhythms in the rat.

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E.4.1.3 EEG registration in conscious cats

PURPOSE AND RATIONALE

The effect of hypnotics on sleep pattern of EEG tracings can be studied in conscious, freely moving cats with chronically implanted electrodes (Heinemann et al. 1970, 1973; Wallach et al. 1976; Hirotsu et al. 1988).

PROCEDURE

Female cats weighing 2.5–3.5 kg are anesthetized and prepared with bipolar subcortical electrodes in the reticular formation (A3, L3, H –1), dorsal hippocampus (A5, L –5, H8), and either amygdala (A12, L –9, H –5), or caudate nucleus (A11, L9.5, H –2). Cortical screw electrodes are placed over the anterior suprasylvian, lateral, medial suprasylvian and ectosylvian gyri. Two Teflon coated steel wires are placed in the cervical neck muscles. All wires are connected to a subminiature socket and implanted in dental acrylic. Cats of this chronic colony are then intermittently utilized for drug experiments at interdrug intervals of at least two weeks.

On experimental days, the cats are taken into an experimental chamber $70 \times 80 \times 80$ cm high. The box is lighted and ventilated with room air at 21 °C. The cat is immediately connected to a cable which exits through the top center of the cage into a mercury swivel. This prevents the cable from becoming twisted and restricting the cat's movement. Recordings of the cortical EEG, cervical neck muscle tone and reticular formation multiple unit activity are obtained. Continuous recordings for up to 96 h are amplified and stored in a recorder. The recordings of cortical EEG, cervical neck muscle tone and reticular formation multiple unit activity are analyzed for REM sleep, slow wave sleep, and wakefulness. Undefined periods which can not be identified either as slow wave sleep or as wakefulness are included in the awake total. Since a first night effect was observed (Wallach et al. 1996) drugs are given at the third or fourth day.

EVALUATION

The data are analyzed by analysis of variance with subjects, days, and drug as factors.

MODIFICATIONS OF THE METHOD

Schallek and Kuehn (1965) measured the effects of benzodiazepines on spontaneous EEG and arousal responses in cats with implanted electrodes.

In addition to EEG and electromyogram, Holm et al. (1991) registered the electro-oculogram in conscious cats.

EEG studies in immobilized cats were performed by Ongini et al. (1982) for evaluation of a benzodiazepine hypnotic. Adult mongrel cats of both sexes were anesthetized with halothane. A tracheal cannula was inserted and artificial respiration was maintained throughout the experiment. The spinal cord was transected at C_2 level (Encephalè isolè preparation). The femoral vein was cannulated for i.v. injection of drugs. Cortical electrodes were inserted into the skull in the frontal, parietal and occipital areas. All incisions were infiltrated with mepivacaine 1% to produce local anes-thesia. The body temperature was maintained at 36.5–38.0 °C by an electrical heat pad. After recovery from surgery and anesthesia, a continuous EEG recording of 2 h was taken prior to drug administration. Test drugs were injected intravenously at various doses. Electrocortical activity was recorded using a 8-channel electroencephalograph. In addition, two electrodes were connected with an EEG-analyzer for the on-line evaluation of the EEG power spectrum. This was computed by the Fast Fourier Transform at a frequency range of 0–32 Hz. Power spectral plots averaging 30 s of electrocortical activity were derived during the experiment.

Shibata et al. (1994) administered various local anesthetics intravenously with constant rates of equipotent doses to cats with implanted electrodes until EEG seizures appeared. During slow rates of infusion, a tetraphasic sequence of changes was found.

Wetzel (1985) evaluated EEG recordings in freely moving **rats** by visual analysis for wakefulness, slow wave sleep or paradoxical sleep.

Krijzer et al. (1991) presented a subclassification of antidepressants based on the quantitative analysis of the electrocorticogram in the rat.

Sarkadi and Inczeffy (1996) described an integrated quantitative electroencephalographic system for pharmacological and toxicological research in the rat. Peak latencies and amplitudes of visual-evoked potentials, occurrence, duration, and linear excursions of photically evoked afterdischarges, activity, mobility, complexity according to Hjorth (1970), and absolute spectral powers of delta, theta, alpha, and beta frequency bands of background activity of visual cortex and frontal-visual leads were measured in freely moving rats.

Rinaldi-Carmona et al. (1992) performed temporal EEG analysis of the sleep-waking cycle in rats with implanted electrodes after administration of a 5-hydroxytryptamine$_2$ receptor antagonist.

Lozito et al. (1994) compared loss of righting with EEG changes in rats with implanted electrodes after single and multiple infusions of fentanyl analogues.

Jones and Greufe (1994) described a quantitative electroencephalographic method in **dogs**.

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E.4.1.4 Automated rat sleep analysis system

PURPOSE AND RATIONALE

Ruigt et al. (1988a,b, 1993) described an automated rat sleep classification system in rats which allows classification of psychotropic drugs such as potential antidepressants, antipsychotics and stimulants (Ruigt and von Proosdij 1990; de Boer and Ruigt 1995).

The system records and analyzes bioelectrical signals from several animals over extended periods of time. The analysis is based on 3 signals, the parieto-occipital EEG, nuchal EMG and a movement indicator signal.

PROCEDURE

Epidural screw electrodes are implanted over the parieto-occipital cortex of male rats weighing 250–300 g for the recording of EEG against a frontal electrode. Stainless-steel wire electrodes are inserted in the dorsal neck musculature for recording the electromyogram (EMG). After recovery from surgery animals are separately housed in a light- (12 : 12 h light-dark cycle) and temperature- (21 °C) controlled room. Twenty-nine hour EEG and EMG recordings are made in sound-attenuated Faraday cages from 32 rats simultaneously. Movements of the rats are detected as capacitative artefacts generated in an open-ended wire of the non-restraining flat cable connecting the rats to a swivel commutator and to amplification and A/D conversion units, which are hooked up through a data controller to a dedicated PDP-11/83 minicomputer system for on-line spectral EEG analysis and data compression.

Off-line sleep staging on a micro VAX is done per 2-s epoch based on 5 spectral EEG band values (1.0–3.0, 3.0–6.0, 6.0–9.0, 9.5–20.0, 20.0–45.0 Hz), the integrated EMG level and the movement level. A first sleep stage assignment per epoch is done by application of a discriminant function to these epoch values. The discriminant function is derived from a discriminant analysis of visually classified representative recording segments from different sleep stages recorded during a separate calibration experiment for each rat. A moving average EEG smoothing procedure and a set of syntactic classification rules are then used to give a final sleep stage assignment to each specific EEG epoch.

Six sleep-wake stages are distinguished including 2 waking stages: (1) active waking characterized by movement, theta activity and high EMG, and (2) quiet waking without movement. Four sleep stages are discriminated: (3) quiet sleep, characterized by EEG spindles; (4) deep slow-wave sleep with prominent delta activity; (5) pre-REM sleep with spindles against a background of theta activity and low EMG, and (6) REM sleep with theta activity and low EMG.

Each experiment consists of 32 rats divided over maximally 4 groups, including various drug treatment groups (generally several doses of the same drug) and always one placebo group. Drug administration is done at the beginning of the light cycle of the rats. After each experiment 2–3 weeks are allowed for wash-out. Drug effects on sleep-waking behavior are assessed on several parameters extracted from the hypnogram, among which percentage time spent in each of the sleep stages per 30-min period and per rat. This gives for each compound a profile of changes over sleep stages and over time.

EVALUATION

Sleep stage-dependent and sleep-independent parts of the EEG power spectrum are defined by a procedure originally developed by Fairchild et al. (1969, 1971, 1975). First, a normal canonical discriminant analysis is done on 4 EEG frequency bands (1–3, 3–6, 6–9, 9.5–20 Hz) from representative segments of only 3 visually classified sleep stages (quiet waking, deep sleep and REM sleep), the sleep stage being the dependent variable. This results in 2 sleep stage-dependent canonical variables covering 100% of the variance in the data set and two residual canonical variables which are independent of sleep stage assignment. These 2 residual variables are subsequently used in a second canonical discriminant analysis in which the

presence or absence of the drug is used as the dependent variable, resulting in a single canonical variable (the drug score) associated with the drug effect on the sleep stage-independent variance of the EEG spectral parameters.

CRITICAL ASSESSMENT OF THE METHOD

According to the author's own judgment, antidepressants, antipsychotics and stimulants can be discriminated from each other and from placebo successfully from each other and from placebo by this method, whereas nootropics classified as placebo. Unfortunately, anxiolytics, hypnotics and anticonvulsants are classified poorly.

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E.5 Neuroleptic activity

E.5.0.1 General considerations

Neuroleptics have been defined as therapeutics effective against schizophrenia. One has to bear in mind that the effect of certain drugs has not been predicted by pharmacological tests but has been found in clinical trials by serendipity. The clinical discoveries were followed by pharmacological studies in many laboratories (Courvoisier 1956).

Various studies have demonstrated the blockade of postsynaptic catecholamine receptors, especially D_2-receptors to be the main mode of action of most neuroleptics. Several *in vitro* methods measure the receptor blockade by neuroleptics.

Pharmacological models in the development of antipsychotic drugs were reviewed by Costall et al. (1991).

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E.5.1 *In vitro* methods

E.5.1.1 D_1 Receptor assay: [^{3}H]-SCH 23390 binding to rat striatal homogenates

PURPOSE AND RATIONALE

Dopamine receptors are the primary targets in the development of drugs for the treatment of schizophrenia, Parkinson's disease and Huntington's chorea (Seeman and Van Tol 1994).

Reviews on dopamine receptors and their subtypes were given by Baldessarini and Tarazi (1996, Missale et al. 1998), and by the NC-IUPHAR subcommittee on dopamine receptors (Schwartz et al. 1998).

Multiple dopamine receptors are known. Two groups are most studied, designated as D_1 and D_2. In the group of D_1-like dopamine receptors the subtypes D_{1A} and D_5/D_{1B} have been described. To D_2-like dopamine receptors belong the D_{2S}, the D_{2L}, the D_3, and the D_4 receptor (Sokoloff et al. 1990; Civelli et al. 1991; Grandy et al. 1991; Van Tol et al. 1991; Lévesque et al. 1992; Baldessarini et al. 1993; Ginrich and Caron 1993; Todd and O'Malley 1993; Waddington and Deveney 1996).

D_1 receptors are positively linked to adenylate cyclase and the D_2 receptor has been shown to be negatively linked to adenylate cyclase. For typical neuroleptic agents, like butyrophenones, a good correlation was found between D_2 receptor binding and clinically effective doses. Atypical neuroleptics, like clozapine, were found to be potent inhibitors of D_1 and D_4 receptor binding, renewing interest in these receptor types. The compound SCH 23390 was found to be selective for the D_1 receptor.

PROCEDURE

Reagents

[N-Methyl-^{3}H] Sch 23 390 (Amersham Lab., specific activity 67–73 Ci/mmol). For IC_{50} determinations, ^{3}H-Sch 23 390 is made up to a concentration of 10 nM and 50 µl is added to each tube. This yields a final concentration of 0.5 nM in the assay.

d-Butaclamol (Ayerst Laboratories). A 1 mM stock solution is made and diluted 1 : 20.

20 µl are added to 3 tubes for the determination of nonspecific binding.

For the test compounds a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assays ranges from 10^{-5} to 10^{-8} M.

Tissue preparation

Male Wistar rats are decapitated, brains rapidly removed, striata dissected and weighed. The striata are homogenized in 100 volumes of 0.05 M Tris buffer, pH 7.7, using a Tekmar homogenizer. The homogenate is centrifuged at 40 000 *g* for 20 min and the final pellet is resuspended in the original volume of 0.05 Tris buffer, pH 7.7, containing physiological ions (NaCl 120 mM, KCl 5 mM, $MgCl_2$ 1 mM and $CaCl_2$ 2 mM).

Assay

50 µl	0.5 M Tris buffer, pH 7.7, containing physiological ions
380 µl	H_2O
20 µl	vehicle or butaclamol or appropriate concentration of test compound
50 µl	^{3}H-SCH 23 390
500 µl	tissue suspension.

The tubes are incubated at 37 °C for 30 min. The assay is stopped by rapid filtration through Whatman GF/B filters using a Brandell cell harvester. The filter strips are then washed 3 times with ice-cold 0.05 M Tris buffer, pH 7.7, and counted in 10 ml Liquiscint scintillation cocktail.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 1 µM d-butaclamol. IC_{50} calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the average of duplicate determinations.

MODIFICATIONS OF THE METHOD

Wamsley et al. (1992) recommended the radioactive form of a dopamine antagonist, [^{3}H]SCH39 166 as ligand for obtaining selective labeling of D_1 receptors.

Sugamori et al. (1998) characterized the compound NNC 01-0012 as a selective and potent D_{1C} receptor antagonist.

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E.5.1.2
D_2 Receptor assay: [^{3}H]-spiroperidol binding

PURPOSE AND RATIONALE

The neuroleptic compound haloperidol has been used as binding ligand to study the activity of other neuroleptics. The use of haloperidol has been superseded by spiroperidol. Dopamine receptor binding assays employing dopaminergic antagonists in mammalian striatal tissue, a dopamine-enriched area of the brain, have been shown to be predictive of *in vivo* dopamine receptor antagonism and antipsychotic activity. Significant correlations exist between neuroleptic binding affinities and their molar potencies in antagonism of apomorphine- or amphetamine-induced stereotypy, apomorphine-induced emesis in dogs, and antipsychotic activity in man. Spiroperidol is considered to be an antagonist specific for D_2 receptors.

PROCEDURE

Tissue preparation

Male Wistar rats are decapitated, their corpora striata removed, weighed and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 40 000 *g* for 15 min. The pellet is rehomogenized in fresh buffer and recentrifuged at 40 000 *g*. The final pellet is then resuspended in Tris buffer containing physiological salts (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$) resulting in a concentration of 10 mg/ml.

Assay

The membrane preparations are incubated with ^{3}H-spiroperidol (0.25 nM) and various concentrations of test drug at 37 °C for 20 min in a K/Na phosphate buffer (50 mM, pH 7.2), followed by cooling in an ice bath for 45 min. To determine non-specific binding, samples containing 10 mM (+)-butaclamol are incubated under identical conditions without the test compound.

Bound ligand is separated by rapid filtration through Whatman GF/B glass fiber filters. The filters are washed three times with ice-cold buffer, dried, and shaken thoroughly with 3.5 ml scintillation fluid. Radioactivity is determined in a liquid scintillation counter. Specific binding is defined as the difference between total binding and the binding in the presence of 2.0 mM (+)-butaclamol.

EVALUATION

The following parameters are determined:

- total binding of ^{3}H-spiroperidol
- non-specific binding: binding of samples containing 2 mM butaclamol
- specific binding = total binding – non-specific binding
- % inhibition: 100 – specific binding as percentage of the control value.

IC_{50} values are determined using at least 3–4 different concentrations of the test compound in triplicate. Results are presented as mean ± standard deviation.

Dissociation constants (K_d) are determined, using ^{3}H-spiroperidol concentrations ranging between 0.1 and 1.0 nM. K_i values (inhibitory constants) are calculated using the following equation:

$$K_i = \frac{IC_{50}}{1 + c/K_d}$$

c = ^{3}H-spiroperidol concentrations used to determine IC_{50}

Standard values: K_i of haloperidol = 6.0 ±1.2 nM

MODIFICATIONS OF THE METHOD

Two isoforms of the D_2 receptor were found by alternative splicing: the long (D_{2L}) and the short (D_{2S}) isoform (Dal Toso et al. 1988; Giros et al. 1989; Monsma et al. 1989; Itokawa et al. 1996).

Niznik et al. (1985) recommended [^{3}H]-YM-09 151-2, a benzamide neuroleptic, as selective ligand for dopamine D_2 receptors.

Hall et al. (1985) used [^{3}H]-eticlopride, a substituted benzamide, selective for dopamine-D_2 receptors, for *in vitro* binding studies.

Radioactive ligands for the D_2 and the D_3 receptor were described by Seeman and Schaus (1991), Chumpradit et al. (1994), Booze and Wallace (1995), Gackenheimer et al. (1995), Seeman and van Tol (1995), Van Vliet et al. (1996).

Vessotskie et al. (1997) characterized binding of [^{125}I]S(–)5-OH-PIPAT to dopamine $D_{2\text{-like}}$ receptors.

Neve et al. (1992) used a special apparatus, the 'cytosensor microphysiometer' which measures the rate of proton excretion from cultured cells (McConnell et al. 1991, 1992; Owicki and Parce 1992). In C_6 glioma cells and L fibroblasts expressing recombinant dopamine D_2 receptors, the dopamine D_2 receptor agonist, quinpirole, accelerated the rate of acidification of the medium dose-dependent up to 100 nM quinpirole. The response was inhibited by the D_2 antagonist spiperone. The D_2 receptor stimulated acidification was due to transport of protons by a Na^+/H^+ antiporter which was verified by the inhibition with amiloride or methylisobutyl-amiloride.

Human recombinant dopamine D_{2A} and D_{2B} receptors

Hayes et al. (1992) described functionally distinct human recombinant subtypes of the dopamine D_2 receptor, D_{2A} and D_{2B}.

D_{2A} receptor binding

In a radioligand binding assay the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D_{2A} receptor is measured.

Twenty mg of membrane is incubated with [^{3}H]-spiperone at a concentration of 2.0 nM for 2 h at 25 °C. Non-specific binding is estimated in the presence of 10 mM haloperidol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

D_{2B} receptor binding

In a radioligand binding assay the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D_{2B} receptor is measured.

Fifteen mg of membrane is incubated with [^{3}H]-spiperone at a concentration of 0.7 nM for 2 h at 37 °C. Nonspecific binding is estimated in the presence of 10 mM haloperidol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

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E.5.1.3 Dopamine D_2 receptor autoradiography (^{3}H-Spiperone binding)

PURPOSE AND RATIONALE

Autoradiography of ^{3}H-spiperone binding sites using selective labeling conditions permits the visualization of the anatomical locations of D_2-dopamine receptors

(Palacios et al. 1981). Quantitative measurements of the binding to receptors can be obtained with computer-assisted video analysis of the autoradiograms with a greater anatomical resolution and sensitivity than in membrane homogenates (Altar et al. 1984, 1985). Using autoradiographic techniques, it has been demonstrated that striatal D_2 receptors are present on intrinsic neurons (Trugman et al. 1986; Joyce and Marshall 1987) and that the distribution of D_2 receptors within the striatum is not homogeneous (Joyce et al. 1985). Anatomically discrete interactions of drugs with D_2 receptors can be examined *in vitro* with inhibition experiments and *ex vivo* following acute or chronic drug treatment of the whole animal.

Since ^{3}H-spiperone labels serotonin-2 (5-HT_2) sites in many brain regions, a masking concentration of a 5-HT_2 receptor blocker, e.g. ketanserin, is included to selectively define binding to D_2 receptors. This is necessary if the test compound inhibits 5-HT_2 binding, or if the brain region of interest has a low D_2 receptor density.

The assay is used to determine potential antipsychotic activity of compounds via direct interaction with the D_2 dopamine recognition site in discrete regions of the rat brain.

PROCEDURE

Reagents

1a. 0.5 M Tris + 1.54 M NaCl, pH 7.4
1b. 0.05 M Tris + 0.154 M NaCl, pH 7.4
2. ^{3}H-spiperone (specific activity 70–90 Ci/mmol) is obtained from Amersham (TRK.818).

   For IC_{50} determinations: ^{3}H-spiperone is prepared at a concentration of 8 nM and 0.55 ml is added to each slide mailer (yields a final concentration of 0.4 nM in the 11.0 ml assay volume).

   For saturation experiments: ^{3}H-spiperone is prepared at a concentration of 20 nM. The final concentrations should range from 0.2–1.0 nM. Typically, six concentrations are used by adding 0.55 ml or less to each mailer (for smaller volumes, add water to bring total addition of 0.55 ml).
3. Sulpiride is obtained from Sigma. A stock solution of 5×10^{-4} M is made by dissolving the sulpiride in 1.0 ml of 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. 0.22 ml of the stock solution is added to the nonspecific binding slide mailers (final concentration 10 µM). All other mailers receive 0.22 ml of vehicle (1 ml of 0.01 N acetic acid in a final volume of 10 ml with distilled water).
4. Ketanserin (free base or tartrate salt) is obtained from Janssen. A stock solution of 10^{-3} M is made by dissolving the ketanserin in 0.5 ml 1 N acetic acid and bringing the final volume to 10 ml with distilled water. The tartrate salt is water-soluble. This is further diluted to 5×10^{-6} M (50 µl q.s. to 10 ml). 0.22 ml is added to all mailers.
5. Test compounds (for IC_{50} determinations). For most assays, a 5×10^{-3} M stock solution is made up in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue preparation

Rat brain sections are collected from plates 9 (rostral nucleus accumbens) through plate 17 (caudal striatum) of "The Rat Brain Atlas in Stereotaxic Coordinates" by Paxinos and Watson.

1. For *in vitro* inhibition experiments, 3–5 sets of 10 slides are collected with 3–4 sections per slide.
2. For saturation experiments, 3–5 sets of 12 slides are collected with 3–4 sections per slide.
3. For *ex vivo* inhibition experiments, a set of 8 slides is used, 4 for total binding and 4 for nonspecific binding.
4. For experiments in which the tissue sections will be swabbed and counted with scintillation fluid, 2 sections per slide are collected.

Assay

1. Preparation of slide mailers (11.0 ml volume/slide mailer):

   Note: If slides with sections are to be wiped for scintillation counting, a final volume of 6.5 ml is sufficient to cover 2 sections. A proportional adjustment of the volumes to be pipetted is made.

a) *In vitro* inhibition experiments

Separate mailers are prepared for total binding, nonspecific binding and 7–8 concentrations of test compound. Ketanserin is included in all mailers to mask binding of [^{3}H]-spiperone to 5-HT_2 sites so that inhibition of binding is D_2-selective.

5.50 ml	buffer 1b
0.55 ml	buffer 1a
0.55 ml	[^{3}H]-spiperone, 0.4 nM final concentration
3.96 ml	distilled water
0.22 ml	ketanserin, 5×10^{-6} M, final concentration 100 nM or vehicle
0.22 ml	test compound, final concentration 10^{-8}–10^{-5} M or sulpiride 5×10^{-4}, final conc. 10 µM or vehicle

b) *Ex vivo* inhibition experiments

Separate mailers are prepared for total and nonspecific binding, as described above, including ketanserin to mask 5-HT_2 receptor binding.

c) Saturation experiments

Separate mailers are prepared for total and non-specific binding at each radioligand concentration. Ketanserin is not included in the mailers, in saturation experiments, since specific binding is defined as sulpiride-displaceable.

5.50 ml buffer 1b
0.55 ml buffer 1a
0.55 ml [^{3}H]-spiperone, final concentration 0.2–1.0 nM
4.18 ml distilled water
0.22 ml 5×10^{-4} M sulpiride, final concentration 10 µM or vehicle

2. Slides are air-dried for 10–15 min at room temperature, preincubated in 0.05 M Tris + 0.154 M NaCl, pH 7.4 for 5 min and further incubated for 60 min with [^{3}H]-spiperone. Slides are then rinsed with ice-cold solutions as follows: dipped in buffer 1b, rinsed in buffer 1b for 2×5 min, and dipped in distilled water.

Slides used for wipes: both sections are wiped with one Whatman GF/B filter and radioactivity is counted after addition of 10 ml of scintillation fluid.

Slides used for autoradiography: slides are dried under a stream of air at room temperature and are stored in a desiccator under vacuum at room temperature (usually over night). Slides are then mounted onto boards, along with ^{3}H-standards (Amersham RPA 506).

In the dark room under safelight illumination (Kodak GBX-2 filter), slides are exposed to Amersham Hyperfilm or LKB Ultrofilm for 14–17 days.

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E.5.1.4 Binding to the D_3 receptor

PURPOSE AND RATIONALE

Sokoloff et al. (1990) reported molecular cloning and characterization of a dopamine receptor (D_3) as a potential target for neuroleptics. The D_3 receptor is localized in limbic areas of the brain which are associated with cognitive, emotional and endocrine functions. Together with the D_{2S}, the D_{2L} and the D_4 receptor, the D_3 receptor belongs to the group of D_2-like dopamine receptors (Ginrich and Caron 1993). 7-[^{3}H]hydroxy-*N*,*N*-di-n-propyl-2-aminotetralin (Lévesque et al. 1992), R(+)-7-OH-DPAT (Baldessarini et al. 1993; and [^{125}I]trans-7-OH-PIPAT-A (Kung et al. 1993) have been recommended as ligands for receptor binding studies.

Chio et al. (1993) compared the heterologously in Chinese hamster ovary cells expressed D_3 dopamine receptors with D_2 receptors.

Damsma et al. (1993) described R-(+)-7-OH-DPAT (R-(+)-7-hydroxy-2-(N,N-di-n-propylamino)tetralin) as a putative dopamine D_3 receptor ligand.

Functional correlates of dopamine D_3 receptor activation in the rat *in vivo* and their modulation by the selective agonist, (+)-S 14 297, have been described by Millan et al. (1995).

Isoforms of the D_3 receptor have been described (Pagliusi et al. 1993).

Akunne et al. (1995) described binding of the selective dopamine D_3 receptor agonist ligand [^{3}H]PD 12 8907 = 4aR,10bR-(+)-trans-3,4,4a,10b-tetrahydro-4-n-propyl-2H,5H-[1]benzopyrano[4,3-b]1,4-oxazin-9-ol.

PROCEDURE

Human dopamine D_3 receptor is expressed in Chinese hamster ovary cells. Cells are grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells are harvested by trypsin treatment (0.25%) for 4–5 min and centrifugation at 2000 *g* for 5 min. They are homogenized with a Polytron in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and are centrifuged at 35 000 *g* for 15 min. The pellet is then resuspended by sonication in a buffer containing 50 mM NaHepes, 1 mM EDTA, 50 µM 8-hydroxyquinoline,

0.005% ascorbic acid, and 0.1% bovine serum albumin (pH 7.5) (incubation buffer). Membrane suspensions (15–25 µg protein) are added to polypropylene test tubes containing [^{3}H]7-OH-DPAT (7-[^{3}H]hydroxy-*N*,*N*-di-n-propyl-2-aminotetralin) for the D_3 receptor assay. Competing drugs are dissolved in incubation buffer, the final volume being 1 ml. Tubes are incubated in triplicate for 1 h at room temperature. The incubations are stopped by rapid filtration under reduced pressure through Whatman GF/C glass filters coated with 0.1% bovine serum albumin, followed by 3 rinses with 3–4 ml ice-cold buffer. Non-specific binding is measured in the presence of 1 µM dopamine.

EVALUATION

Saturation curves are analyzed by computer non-linear regression using a one-site cooperative model to obtain equilibrium dissociation constants (K_D) and maximal density of receptors (B_{max}). Inhibition constants (K_i) are estimated according to the equation

$$K_i = IC_{50} / 1 + L / K_D$$

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E.5.1.5 Binding to D_4 receptors

PURPOSE AND RATIONALE

Van Tol et al. (1991) reported cloning of the gene of a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. Together with the D_{2S}, the D_{2L} and the D_3 receptor, the D_4 receptor belongs to the group of D_2-like dopamine receptors (Ginrich and Caron 1993). Recognition and characterization of this dopamine binding site may be useful in the design of new types of antipsychotic drugs.

Dopamine D_4 receptors have been localized in GABAergic neurons of the primate brain (Mrzljak et al. 1996).

PROCEDURE

A plasmid construct of a 3.9-kb gene-cDNA hybrid subcloned into the expression vector pCD-PS is introduced into COS-7 cells by calcium phosphate mediated transfection. Cells are cultivated and homogenized (Teflon pestle) in 50 mM Tris-HCl (pH 7.4 at 4 °C) buffer containing 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM KCl, and 120 mM NaCl. Homogenates are centrifuged for 15 min at 39 000 *g*, and the resulting pellets resuspended in buffer at a concentration of 150–250 µg/ml. For saturation experiments, 0.25 ml of tissue homogenate are incubated in duplicate with increasing concentrations of [^{3}H]spiperone (70.3 Ci mmol^{-1}; 10–3 000 pM final concentration) for 120 min at 22 °C in a total volume of 1 ml. For competition binding experiments, assays are initiated by the addition of 0.25 ml membrane and incubated in duplicate with various concentrations

of competing ligands (10^{-14}–10^{-3} M) and [^{3}H]spiperone (150–300 pM) either in the absence or the presence of 200 µM Gpp(NH)p for 120 min at 22 °C. Assays are terminated by rapid filtration through a Titertek cell harvester and filters then monitored for tritium. For all experiments, specific binding is defined as that inhibited by 10 µM (–)sulpiride.

EVALUATION

Both saturation and competition binding data are analyzed by the non-linear least-square curve-fitting program LIGAND run on a suitable PC.

MODIFICATIONS OF THE METHOD

Human recombinant dopamine $D_{4,2}$, $D_{4,4}$, $D_{4,7}$, and D_5 receptors

Van Tol et al. (1992) described multiple dopamine D_4 receptor variants in the human population.

Sunahara et al. (1991) reported the cloning of the gene for a human D_5 receptor.

Human recombinant dopamine $D_{4,2}$ receptor binding

In a radioligand binding assay the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine $D_{4,2}$ re-ceptor is measured.

Fifteen µg of membrane is incubated with [^{3}H]-spiperone at a concentration of 0.7 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 µM haloperidol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [^{3}H]- spiperone bound.

Human recombinant dopamine $D_{4,4}$ receptor binding

In a radioligand binding assay the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine $D_{4,4}$ receptor is measured.

Twenty-five µg of membrane are incubated with [^{3}H]-spiperone at a concentration of 1.0 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 µM haloperidol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

Human recombinant dopamine $D_{4,7}$ receptor binding

In a radioligand binding assay the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine $D_{4,7}$ receptor is measured.

Fifteen µg of membrane is incubated with[^{3}H]-spiperone at a concentration of 0.7 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 µM haloperidol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

Human recombinant dopamine D_5 receptor

In a radioligand binding assay the binding of [^{3}H]SCH 23 390 to membranes prepared from COS cells expressing a recombinant human dopamine D_5 receptor is measured.

Forty µg of membrane is incubated with [^{3}H]SCH 23 390 at a concentration of 2 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 µM *cis*-flupentixol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [^{3}H]SCH 23 390 bound.

Several selective dopamine D_4 antagonists were described: Hikada et al. (1996), Merchant et al. (1996), Rowley et al. (1996), Bristow et al. (1997).

Some radioligands were proposed as being selective for dopamine D_4 receptors: [^{3}H]clozapine (Ricci et al. 1997a,b), [^{3}H]NGD 94-1 (Thurkauf 1997; Primus et al. 1997), RBI-257 (Kula et al. 1997).

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E.5.1.6 Determination of dopamine autoreceptor activity

PURPOSE AND RATIONALE

The method describes the procedure to determine if a compound possesses autoreceptor blocking activity without the interference from postsynaptic effects. Striatal DOPA (3,4-dihydroxyphenylalanine), DOPAC (3,4-dihydroxyphenylacetic acid) and DA (dopamine) are quantitated following *in vivo* treatment with drug, apomorphine, gamma butyrolactone and NSD-1015. Antipsychotic compounds that block striatal dopaminergic presynaptic autoreceptors are believed to possess a greater liability for producing EPS.

PROCEDURE

Reagents

1. 0.1 M HCl
2. 1 N NaOH
3. 0.1 M perchloric acid (PCA) containing 4.3 mM EDTA
4. 2 mM solutions of DOPAC, DA and DOPA in 0.1 M HCl, 0.5 ml aliquots are stored at –60 °C until use.
5. Preparation of 2° standard mixture
   10 µM solution of DOPAC, DA and DOPA diluted from reagent 4 with 0.1 M PCA/EDTA);
   The 2° standard solution is used for the preparation of standard curves.
6. Mobile phase/MeOH-buffer (4 : 96, v/v) buffer: 0.012 mM sodium acetate, 0.036 M citric acid and 152 µM sodium octane sulfonate, mobile phase: methanol/buffer (80 ml + 1920 ml) is filtered through a 0.2 µm nylon 66 filter.
7. Preparation of dosing solutions
   a) Apomorphine (2 mg/kg) is prepared in saline containing 1% Tween 80 + 0.1% ascorbic acid to prevent oxidation.
   b) GBL (750 mg/kg) is prepared as a solution in saline containing 1% Tween 80.
   c) NSD-1 015 (100 mg/kg) is prepared as a solution in saline containing 1% Tween 80.

HPLC-Instrumentation

Consists of a

- pump, model SP8 810 (Spectra Physics),
- injector, WISP 710B (Waters Associates),
- detector, 5 100A electrochemical with a 5 011 analytical cell and 5 020 guard cell (ESA),
- integrator, D-2 000 (Hitachi), used as a back-up for the data collection/integrator, CS 9 000 (IBM) system
- analytical column: C18-ODS Hypersil, 3 µm, 100 × 4.6 mm (Shandon)

Tissue Preparation

Following treatment with test drug, rats are sacrificed by decapitation at the pre-determined time. The brain is rapidly removed, the striatum is dissected on ice and frozen on dry ice. The tissue is analyzed by HPLC the same day.

Tissue is homogenized in 500 µl 0.1 M PCA/EDTA. The homogenate is centrifuged for 6 min using a microcentrifuge (model 5 413, Eppendorf). The supernatant is transferred to 0.2 µm microfilterfuge™ tubes and cen-trifuged for 6–8 min as before. The filtrate is transferred to WISP vials. Standards are included every 12–15 samples.

Five µl of the striatum homogenate is injected into the HPLC column.

HPLC flow rate is 1.5 ml/min, run time is 20 min. Helium flow is constant in mobile phase.

For protein analysis, 1.0 ml 1 N NaOH is added to the tissue pellet. The next day, the protein analysis is performed as described by Bradford (1976) using the BioRad assay kit.

EVALUATION

Peak area is used for quantitation. The mg of protein and pmoles of DOPAC, DA and DOPA are calculated from linear regression analyses using the corresponding standard curve. Final data are reported as pmoles/mg protein.

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E.5.1.7 Dopamine-sensitive adenylate cyclase in rat striatum

PURPOSE AND RATIONALE

Agonist stimulation of dopamine D_1 receptors leads to increased cAMP formation mediated by a guanine nucleotide-binding regulatory protein. This effect is blocked by selective antagonists like SCH 23 390.

Agonist stimulation of the dopamine D_2 receptor leads to a decreased cAMP formation mediated by a gua-nine nucleotide binding protein. Apomorphine is a potent agonist with full intrinsic activity at D_2 receptors. Phenothiazines block both D_1 and D_2 receptors, whereas butyrophenones and related drugs are very potent antagonists at D_2 receptors.

Studies on cAMP formation may be useful for differentiation of antipsychotic drugs.

PROCEDURE

Tissue preparation

Male Wistar rats are sacrificed by decapitation, the brains removed, and the striata dissected out and weighed. Striatal tissue from 2 rats is homogenized in 25 volumes of ice-cold 0.08 M Tris-maleate buffer, pH 7.4, containing 2 mM EGTA. Protein content of an aliquot is determined. A 50 µl aliquot is used in the cyclase enzyme assay.

Enzyme assay

The following volumes are placed in conical centrifuge tubes kept in an ice-water bath:

200 µl incubation buffer (equal amounts of 0.8 mM Tris-maleate, pH 7.4; 60 mM $MgSO_4$; 100 mM theophylline and 4 mM EGTA)
50 µl 1 mM dopamine HCl or water
25 µl test drug or water
125 µl distilled water
50 µl tissue homogenate

After incubation for 20 min at 0 °C, the enzyme reaction is started by addition of 50 µl of 15 mM ATP solution. The tube rack is placed in a shaking water bath preset at 30 °C for 2.5 min. The reaction is terminated by placing the tube rack in a boiling water bath for 4 min. Then, the tubes are centrifuged at 1 000 *g* for 10 min.

A 25 µl aliquot of the supernatant in each tube is removed and the cAMP determined using a commercial RIA kit (Amersham).

EVALUATION

Results are expressed as pmoles cAMP/mg protein of dopamine stimulated vs. non dopamine stimulated level. Percentage inhibition of this dopamine stimulated level by test drugs is calculated and IC_{50} values determined by log-probit analysis.

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E.5.1.8 α_1-adrenergic receptor binding in brain

PURPOSE AND RATIONALE

The use of neuroleptic and antidepressant drugs is sometimes limited by their side effects, such as orthostatic hypotension and sedation. These side effects are attributed to blockade of central and peripheral adrenergic α-receptors. For neuroleptics the ratio between their dopamine antagonistic and their α-receptor antagonistic potencies should be taken into account rather than their absolute α-blocking effect. WB-4 101 is a specific and potent antagonist of the α_1-adreno-receptor, characterized *in vitro* in rat brain, heart, vascular smooth muscle and gastrointestinal smooth muscle.

The *in vitro* [^{3}H]-WB 4 101 receptor binding assay quantitates the α-adrenergic blocking properties of psychoactive agents and is used to assess a compound's potential to cause orthostatic hypotension and sedation as well as primary blood pressure lowering effects through α_1-receptor blockade.

PROCEDURE

Reagents

[Phenoxy-3-^{3}H(N)]-WB 4101 = (2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane, New England Nuclear, (specific activity 20–35 Ci/mmol).

For IC_{50} determinations [^{3}H]-WB 4 101 is made up to a concentration of 2 nM in Tris buffer and 500 µl is added to each tube (yields a final concentration of 0.5 nM in the 2 ml assay)

L-norepinephrine bitartrate (Sigma Chemical Company). A 800 µM solution is prepared in Tris buffer and 250 µl is added to each of 3 tubes to determine nonspecific binding. This yields a final concentration of 100 µM in the 2 ml assay.

Test compounds: A 80 µM stock solution is made up in a suitable solvent and serially diluted with Tris buffer, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Usually, seven concentrations are studied for each assay.

Tissue preparation

Male Wistar rats (100–150 g) are sacrificed by decapitation. The whole brain minus cerebellum is homogenized in 75 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 40 000 *g* at 4 °C for 15 min. The supernatant is discarded and the pellet is rehomogenized in fresh Tris buffer and recentrifuged at 40 000 *g* at 4 °C for 15 min. The final pellet is resuspended in the original volume of ice-cold 0.05 M Tris buffer. The final tissue concentration in the assay is 10 mg/ml. Specific binding is approximately 80% of total bound ligand.

Assay

1 200	µl	tissue suspension
500	µl	^{3}H-WB 4 101
250	µl	vehicle (for total binding) or
800	µM	L-norepinephrine bitartrate (for nonspecific binding) or appropriate drug concentration

Sample tubes are kept in ice for additions, then vortexed and incubated for 15 min at 25 °C. The binding is terminated by rapid vacuum filtration through Whatman GF/B filters, followed by three 5 ml washes with ice-cold 0.05 M Tris buffer. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

EVALUATION

Specific WB 4 101 binding is defined as the difference between the total binding and that bound in the presence of 100 µM norepinephrine. IC_{50} calculations are performed using computer-derived log-probit analysis.

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E.5.1.9 [^{3}H]Spiroperidol binding to 5-HT$_2$ receptors in rat cerebral cortex

PURPOSE AND RATIONALE

The purpose of this assay is to determine the anti-serotonin activity of neuroleptics, antidepressants and antihypertensive compounds, by measuring the displacement of [^{3}H]spiroperidol from serotonergic antagonist binding sites in cerebral cortical membranes. The regulation of 5-HT$_2$ receptor density by chronic antidepressant treatment is discussed in a separate protocol (see Sect. E.6.1.6).

The receptor binding of serotoninergic sites in the CNS has been investigated using [^{3}H]serotonin (5-HT) (Bennett and Snyder 1976), [^{3}H]LSD (Peroutka and Snyder 1979) and [^{3}H]spiroperidol (Peroutka and Snyder 1979; List and Seeman 1981; Leysen et al. 1978) as the radioligand. Receptor sites have been defined kinetically and classified as 5-HT$_1$ sites (labeled by [^{3}H]5-HT, displaced by agonists) and 5-HT$_2$ sites (labeled by [^{3}H]-spiroperidol and displaced by antagonists). [^{3}H]LSD labels both 5-HT$_1$ and 5-HT$_2$ binding sites (Peroutka and Snyder 1979). Of the brain regions tested, the frontal cerebral cortex contained the greatest density of 5-HT$_2$ binding sites. Lesioning studies indicate that 5-HT$_2$ binding sites are post synaptic and not linked to adenylate cyclase (Peroutka et al. 1979).

The inhibition of 5-HT$_2$ binding correlates with the inhibition of quipazine-induced head twitch, which may reflect decreased behavioral excitation. The physiological and pharmacological role of these receptors is not clear. Although numerous neuroleptics and antidepressants of varying chemical structures are potent inhibitors of 5-HT$_2$ binding, there is no clear-cut relationship to the efficacy of these drugs. Methysergide and cyproheptadine are both potent inhibitors of 5-HT$_2$ binding without having neuroleptic or antidepressant effects. However, potent interaction with 5-HT$_2$ receptors may indicate a reduced potential for catalepsy, since methy-sergide blocks catalepsy induced by haloperidol (Rastogi et al. 1981). The interaction of serotonergic neurons with cholinergic neurons in the striatum (Samanin et al. 1978) may also be decreased by potent 5-HT$_2$ antago-nists. In addition, the ratio of activity at D$_2$ and 5-HT$_2$ re-ceptors may be useful in the screening of atypical antipsychotic agents (Meltzer et al. 1989). Furthermore, it has been shown that ketanserin, a selective 5-HT$_2$ antagonist, is an effective hypotensive agent which blocks peripheral vascular 5-HT receptors.

5-HT$_2$ receptors have been subdivided into 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors. The new 5-HT receptor classification has been published by the VII. International Union of Pharmacology Classification of Receptors for 5-Hydroxytryptamine (Serotonin) (Hoyer et al. 1994). Further comments were given by Humphrey et al. (1993), Martin and Humphrey (1994), Saxena (1994), Tricklebank (1996).

PROCEDURE

Reagents

1. 0.5 M Tris buffer, pH 7.7
   a) 57.2 g Tris HCl
      16.2 g Tris base
      q.s. to 1 liter (0.5 M Tris buffer, pH 7.7)
   b) Make a 1 : 10 dilution in distilled H_2O
      (0.05 M Tris buffer, pH 7.7)
2. Tris buffer containing physiological ions
   a) Stock buffer

NaCl	7.014 g
KCl	0.372 g
$CaCl_2$	0.222 g
$MgCl_2$	0.204 g

   q.s. to 100 ml in 0.5 M Tris buffer
   b) Dilute 1 : 10 in distilled H_2O
      This yields 0.05 M Tris HCl, pH 7.7; containing NaCl (120 mM), KCl (5 mM), $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM)
3. [Benzene-^{3}H] spiroperidol (20–35 Ci/mmol) is obtained from New England Nuclear. For IC_{50} determinations: ^{3}H-spiroperidol is made up to a concentration of 30 nM in 0.01 N HCl and 50 µl added to each tube (yields a final concentration of 1.5 nM in the 1 ml assay).
4. Methysergide maleate is obtained from Sandoz. Methysergide maleate stock solution is made up to 0.25 mM for determination of nonspecific binding.
   The final concentration in the assay is 5 µM, when 20 µl of the stock solution is added to the reaction tube.
5. Test compounds. For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay and higher or lower concentrations may be used, depending on the potency of the drug.

Tissue preparation

Male Wistar rats are decapitated and the cerebral cortical tissue is dissected, weighed and homogenized in 50 volumes of 0.05 M Tris buffer, pH 7.7 (buffer 1b) with the Brinkman Polytron, then centrifuged at 40 000 *g* for 15 min. The supernatant is discarded and the pellet resuspended and recentrifuged as described above. This pellet is resuspended in 50 volumes of buffer 2b and stored in an ice bath. The final tissue concentration is

10 mg/ml. Specific binding is 7% of the total added ligand and 50% of total bound ligand.

Assay

50 µl 0.5 M Tris-physiological salts (buffer 2a)
380 µl H_2O
20 µl vehicle (for total binding) or 0.25 mM methysergide (for nonspecific binding) or appropriate drug concentration
50 µl [^{3}H]spiroperidol
500 µl tissue suspension

The samples are incubated for 10 min at 37 °C, then immediately filtered under reduced pressure using Whatman GF/B filters. The filters are washed with three 5 ml volumes of ice-cold 0.05 M Tris buffer, pH 7.7 mM methysergide.

EVALUATION

IC_{50} calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

MODIFICATION OF THE METHOD

The receptor binding properties of the 5-HT_2 antagonist ritanserin were reported by Leysen et al. (1985).

Preclinical characterization of a putative antipsychotic as a potent 5-HT_{2A} antagonist was reported by Kehne et al. (1996).

Using [^{125}I]LSD and [^{3}H]5-HT binding assays Siegel et al. (1996) characterized a structural class of 5-HT_2 receptor ligands.

[^{3}H]Ketanserin has been described as a selective ^{3}H-ligand for 5-HT_2 receptor binding sites. (Leysen et al. 1981).

[^{3}H]RP 62 203, a potent and selective 5-HT_2 antagonist was recommended for *in vivo* labeling of 5-HT_2 receptors (Fajolles et al. 1992).

Other selective 5-HT_2 receptor radioligands were recommended:

[^{125}I]-EIL (radioiodinated D-(+)-N1-ethyl-2-iodolysergic acid diethylamide) (Lever et al. 1991); [^{3}H]-MDL100,907 (Lopez-Gimenez et al. 1998).

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E.5.1.10 Serotonin 5-HT$_2$ receptor autoradiography (^{3}H-Spiperone binding)

PURPOSE AND RATIONALE

Autoradiography of ^{3}H-spiperone binding sites with selective labeling conditions permits the visualization of the anatomical locations of 5-HT$_2$ receptors (Palacios et al. 1981; Pazos et al. 1985; Altar et al. (1985). Quantitative measurements of the binding to receptors can be obtained with computer-assisted video analysis of the autoradiograms with a greater anatomical resolution and sensitivity than in membrane homogenates (Pazos et al. 1985; Altar et al. (1984). Using autoradiographic techniques, it has been demonstrated that there is a heterogeneous distribution of 5-HT$_2$ receptors, with much higher levels in telencephalic areas such as the neocortex and the claustrum than in meso- or metencephalic areas. Within the cortex, 5-HT$_2$ receptors are abundant in layers IV and V (Pazos et al. 1985). The high concentration of 5-HT$_2$ receptors in the frontoparietal motor area and the claustrum which connects to the motor cortex and other motor areas suggests a physiological role for 5-HT$_2$ receptors in some motor syndromes (Cadet et al. 1987; Costall et al. 1975; Kostowski et al. 1972). The high affinity of the atypical antipsychotic clozapine for 5-HT$_2$ receptors (Fink et al. 1984; Altar et al1986) and the down-regulation of 5-HT$_2$ receptors following chronic administration of clozapine (Reynolds et al. 1983; Lee and Tang 1984; Wilmot and Szczepanik 1989) suggests that 5-HT$_2$ receptor interaction may be a significant factor in the lack of extrapyramidal side effects and tardive dyskinesias with its clinical use.

Since ^{3}H-spiperone labels α_1-noradrenergic sites in the cerebral cortex, a masking concentration of the α_1-blocker prazosin is included to selectively define binding to 5-HT$_2$ receptors (Morgan et al. 1984). This is necessary if the test compound also inhibits α_1-receptors which may be present in the brain region of interest.

The assay is used to determine the direct interaction of potential antipsychotic compounds with the serotonin-5-HT$_2$ recognition site in discrete regions of the rat brain either *in vitro* or after *ex vivo* treatment of the whole animal.

PROCEDURE

Reagents

1a. 0.5 M Tris + 1.54 M NaCl, pH 7.4
1b. 0.05 M Tris + 0.154 M NaCl, pH 7.4
2. ^{3}H-spiperone (specific activity 70–90 Ci/mmol) is obtained from Amersham.
   - For IC_{50} determinations: ^{3}H-spiperone is made up to a concentration of 20 nM and 0.55 ml is added to each slide mailer (yields a final concentration of 1.0 nM in the 11.0 ml assay volume).
   - For saturation experiments: ^{3}H-spiperone is made up to a concentration of 20 nM. The final concentration should range from 0.5–2.5 nM. Typically, six concentrations are used by adding 0.55 ml or less to each mailer (for smaller volumes, add water to bring total addition of 0.55 ml).
3. Methysergide is used to determine nonspecific binding in brain sections of the frontal cortex.

   Methysergide maleate is obtained from Sandoz. A stock solution of 2.5×10^{-4} M is made by dissolving in distilled water. A volume of 0.22 ml of the stock solution is added to the nonspecific binding slide mailers (final concentration 5 µM). All other mailers receive 0.22 ml of vehicle (1 ml of 0.01 N acetic acid in a final volume of 10 ml with distilled water).

4. Ketanserin is used to determine nonspecific binding in those slide mailers containing sections with the nucleus accumbens and striatum.

   Ketanserin (free base or tartrate salt) is obtained from Janssen. A stock solution of 10^{-3} M is made by dissolving the ketanserin (free base) in 0.05 N acetic acid or the tartrate salt in distilled water. This is further diluted to 5×10^{-6} M (50 µM q.s 10 ml with distilled water). A volume of 0.22 ml is added to the slide mailers to give a final concentration of 100 nM.
5. Prazosin is used to mask α_1-receptors in cortical brain section.

   Prazosin HCl is obtained from Pfizer. A stock solution of 10^{-4} M is made by dissolving prazosin in 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. This is further diluted to 5×10^{-6} M (100 µM q.s 10 ml). A volume of 0.22 ml is added to those slide mailers to be used for cortical brain sections to give a final concentration of 100 nM.
6. Sulpiride is used to mask D_2 receptor binding in brain sections from the nucleus accumbens and striatum.

   Sulpiride is obtained from Sigma. A stock solution of 10^{-4} M is made by dissolving sulpiride in 1.0 ml of 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. A volume of 0.22 ml is added to the appropriate slide mailers to give a final concentration of 10 µM.
7. Test compounds (for *in vitro* IC_{50} determinations).

   For most assays, a 5×10^{-3} M stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Frontal cortical brain sections are collected from plates 5 through 8 and nucleus accumbens/striatal sections are collected from plates 9 (rostral n. accumbens) through plate 17 (caudal striatum) of "The Rat Brain Atlas in Stereotaxic Coordinates" by Paxinos and Watson.

1. For *in vitro* inhibition experiments, 3–5 sets of 10 slides are collected with 4–5 sections per slide.
2. For saturation experiments, 3–5 sets of 12 slides are collected with 4–5 sections per slide.
3. For *ex vivo* inhibition experiments, a set of 8 slides is used, 4 for total binding and 4 for nonspecific binding.
4. For experiments in which the tissue sections will be swabbed and counted with scintillation fluid, two sections per slide are collected.

Assay

1. Preparation of slide mailers (11.0 ml volume/slide mailer):

   Note: If slides with sections are to be wiped for scintillation counting, a final volume of 6.5 ml is sufficient to cover 2 sections. A proportional adjustment of the volumes to be pipetted is made.

a) *In vitro* inhibition experiments

   Separate mailers are prepared for total binding, nonspecific binding and 7–8 concentrations of test compound.

   1. For frontal cortical brain sections, prazosin is included in all mailers to mask the binding of [^{3}H]-spiperone to α_1-receptors and nonspecific binding is defined with 5 µM methysergide.
      - 5.50 ml buffer 1b
      - 0.55 ml buffer 1a
      - 0.55 ml [^{3}H]-spiperone, 1.0 nM final concentration
      - 3.96 ml distilled water
      - 0.22 ml prazosin 5×10^{-6} M, final concentration 100 nM or vehicle
      - 0.22 ml test compound, final concentration 10^{-8}–10^{-5} M or methysergide 2.5×10^{-4} M, final concentration 5 µM or vehicle
   2. For brain sections with the nucleus accumbens and striatum in which there is negligible binding of [^{3}H]-spiperone to α_1-receptors, prazosin is not included. Since levels of 5-HT_2 receptors in these brain areas are low, 10 µM sulpiride is included in all mailers to mask the binding of [^{3}H]-spiperone to D_2 receptors.

      Ketanserin, final concentration of 100 nM, is used to determine nonspecific binding since methysergide has a weak affinity for D_2 receptors (IC_{50} approximately 1–5 µM).
      - 5.50 ml buffer 1b
      - 0.55 ml buffer 1a
      - 0.55 ml [^{3}H]-spiperone, 1.0 nM final concentration
      - 3.96 ml distilled water
      - 0.22 ml sulpiride 5×10^{-4} M, final concentration 10 µM or vehicle
      - 0.22 ml test compound, final concentration 10^{-8}–10^{-5} M or ketanserin 5×10^{-5} M final concentration 100 nM or vehicle

b) *Ex vivo* inhibition experiments

   Separate mailers are prepared for total and nonspecific binding, as described above, including sulpiride to mask D_2 receptor binding with brain sections through the nucleus accumbens and striatum and prazosin to mask α_1-receptors in cortical brain sections.

c) Saturation experiments

Separate mailers are prepared for total and nonspecific binding at each radioligand concentration. Prazosin is not included in the mailers in saturation experiments, since specific binding is defined by methysergide which has negligible affinity for α_1-receptors.

5.50 ml buffer 1b
0.55 ml buffer 1a
0.55 ml [^{3}H]-spiperone, final concentrations 0.5–2.5 nM
4.18 ml distilled water
0.22 ml 2.5×10^{-4} M methysergide, final concentration 5 µM or vehicle

2. Slides are air-dried for 10–15 min at room temperature, preincubated in 0.05 M Tris + 0.154 M NaCl, pH 7.4 for 5 min and further incubated for 60 min with [^{3}H]-spiperone. Slides are then rinsed with ice-cold solutions as follows: dipped in buffer 1b, 2×5 min rinsed in buffer 1b, dipped in distilled water.

Slides used for wipes: both sections are wiped with one Whatman GF/B filter and radioactivity is counted after addition of 10 ml of scintillation fluid.

Slides used for autoradiography: slides are dried under a stream of air at room temperature and are stored in a desiccator under vacuum at room temperature (usually overnight). Slides are then mounted onto boards, along with ^{3}H-standards (Amersham RPA 506).

In the dark room under safelight illumination (Kodak GBX-2 filter), slides are exposed to Amersham Hyperfilm or LKB Ultrofilm for 14–17 days.

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E.5.1.11 Binding to the sigma receptor

PURPOSE AND RATIONALE

Sigma receptors, as a class of binding sites in the brain, were originally described as a subtype of the opiate receptors. Efforts to develop less addicting opiate analgesics led to the study of several benzomorphan derivatives which produce analgesia without causing the classical morphine-induced euphoria. Unfortunately, these compounds, like N-allylnormetazocine (SKF 10,047) produced a variety of psychotic symptoms. This psychotomimetic effect is thought to be mediated by sigma receptors. This binding site is sensitive to many neuroleptics, most notably the typical antipsychotic haloperidol, leading to the hypothesis that drug interactions with the sigma site may be a new approach for the discovery of novel antipsychotics which are not dopamine receptor antagonists. D_2 receptor antagonism is thought to be linked with the occurrence of extrapyramidal symptoms in the form of hyperkinesia and Parkinson symptoms or tardive dyskinesia limiting the therapeutic use of traditional antipsychotic medication. It is hoped that ligands to the sigma receptor do not produce these adverse reactions. The sigma site is believed to be distinct from the binding site for the psychotomimetic drug phencyclidine.

PROCEDURE

Reagents

(+)-SKF 10,047 is prepared as a stock solution of 5×10^{-3} M with distilled water. 130 µl added to the 6.5 ml assay yields a final concentration of 10^{-4} M.

^{3}H-(+)-SKF 10,047 (specific activity 40 Ci/mmol) is obtained from New England Nuclear. A 200 nM stock solution is made up with distilled water for IC_{50} determinations. 325 µl added to each tube yields a final concentration of 10 nM in the 6.5 ml assay.

Test compounds

A 5 mM stock solution is prepared in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M.

Tissue preparation

The assay utilizes slide-mounted cross-sections of brain tissue from male Hartley guinea pigs. Whole brain sections of 10 µm thickness are obtained from the hippocampus, thaw-mounted onto gel-chrome alum subbed slides, freeze-dried and stored at –70 °C until use. On the day of the assay, the sections are thawed briefly at room temperature until the slides are dry and then used in the assay at a final volume of 6.5 ml.

Assay

Incubation solutions are prepared in plastic slide mailer containers as follows:

3.250 ml 0.05 M Tris buffer, pH 7.7
2.470 ml distilled water
0.325 ml 0.5 M Tris buffer, pH 7.7
0.130 ml (+)-SKF 10,047 or vehicle
0.325 ml [^{3}H](+)-SKF 10,047

Dried slides with tissue sections are added to the slide mailers and incubated at room temperature for 90 min. Non-bound radioligand is removed by rinsing the slides sequentially in two 5-min rinses in ice-cold 0.05 M Tris buffer and a dip in ice-cold distilled water. The sections are either swabbed with Whatman GF/B filters for scintillation counting of tissue-bound radioligand or exposed to tritium-sensitive film for autoradiography of the binding sites.

EVALUATION

Specific binding is determined from the difference of binding in the absence or presence of 10^{-4} M (+)-SKF 10,047 and is typically 60–70% of total binding. IC_{50} values for the competing drug are calculated by log-probit analysis of the data.

MODIFICATIONS OF THE METHOD

[^{3}H]-(+)-pentazocine has been recommended as a highly potent and selective radioligand for µ receptors (de Costa et al. 1989; DeHaven-Hudkins et al. 1992).

Classification of sigma binding sites into σ_1 and σ_2 receptors has been proposed (Walker et al. 1990; Quirion et al. 1992; Abou-Gharbia et al. 1993).

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E.5.1.12 Simultaneous determination of norepinephrine, dopamine, DOPAC, HVA, HIAA, and 5-HT from rat brain areas

PURPOSE AND RATIONALE

To measure the effects of potential antipsychotic drugs on catecholamines and indols, a quantitative method for the determination of norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindolacetic acid (5HIAA) and 5-hydroxytryptamine (5-HT) from rat brain regions is used. These catecholamines and indols are measured in rat brain prefrontal cortex, nucleus accumbens and striatum.

PROCEDURE

Reagents

1. 0.1 M HCl
2. 1 N NaOH
3. 2 mM solutions of NE, DOPAC, DA, HVA, 5HIAA and 5-HT in 0.1 M HCl;
   0.5 ml aliquots are stored at –60 °C until use.
4. Preparation of 2° Standard Mixture
   - 10 µM solution of NE, DOPAC, DA, HVA, 5HIAA and 5-HT (diluted from reagent 3) in mobile phase (reagent 5)
   - the 2° Standard solution is used for the preparation of standard curves.
5. Mobile phase/MeOH: buffer (7.5 : 92.5, v/v)
   - buffer: 0.07 M sodium acetate, 0.04 M citric acid, 130 µM EDTA and 230 µM sodium octane sulfonate
   - mobile phase: methanol/buffer (150 ml + 1 850 ml) is filtered through a 0.2 µm nylon 66 filter

HPLC-Instrumentation

- pump, model SP8 810 (Spectra Physics),
- injector, WISP 710B (Waters Associates),
- detector, 5 100A electrochemical with a 5 011 analytical cell and 5 020 guard cell (ESA),
- integrator, D-2 000 (Hitachi), used as a back-up for the data collection/integrator, CS 9 000 (IBM) system,
- analytical column: C18-ODS Hypersil, 3 µm, 100 × 4.6 mm (Shandon)

Animal treatment

Six rats per group (150–250 g) are dosed with 4–5 different concentrations of the putative antipsychotic drug; usual concentrations range from 0.03 to 30 mg/kg. At a predetermined time, usually 60 min, the rats are sacrificed.

Tissue preparation

Following treatment with test drug, rats are sacrificed by decapitation. The brain is rapidly removed and placed on ice. The striatum, nucleus accumbens and/or prefrontal cortex are dissected and placed in 1.5 ml microcentrifuge tubes. The tubes are capped and immediately placed in dry ice. The frozen brain sections are stored at –60 °C until HPLC analysis.

Tissue is homogenized in mobile phase (striatum: in 600 µl, nucleus accumbens and prefrontal cortex: in 300 µl). The homogenates are centrifuged for 6 min using a microcentrifuge (model 5 413, Eppendorf). The supernatants are transferred to 0.2 µm microfilterfuge™ tubes and centrifuged for 6–8 min as before. The filtrate is transferred to WISP vials. Standards are included every 12–15 samples.

The following volumes are injected to the HPLC column:

- striatum: 5 µl; nucleus accumbens: 20 µl; prefrontal cortex: 50 µl.
- HPLC flow rate is 1.0 ml/min, run time is 25 min. Helium flow is constant in mobile phase.

For protein analysis, 1 N NaOH is added to the tissue pellets as follows:

- striatum: 1.0 ml;
- nucleus accumbens and prefrontal cortex: 0.5 ml.

The next day, the protein analysis is run in duplicate with 5 µl of striatum. 20 µl of nucleus accumbens and prefrontal cortex as described by Bradford (1976) using the BioRad assay kit.

EVALUATION

Peak area is used for quantitation. The mg of protein and pmoles of NE, DOPAC, DA, HVA, 5HIAA and 5-HT are calculated from linear regression analysis using the corresponding standard curve. Final data are reported as pmoles/mg protein.

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E.5.1.13 Measurement of neurotransmitters by intracranial microdialysis

PURPOSE AND RATIONALE

Methods to measure neurotransmitters and their metabolites in specific areas of the brain by microdialysis were introduced by Ungerstedt and his group (Ungerstedt et al. 1982; Zetterström et al. 1982, 1983; Zetterström and Ungerstedt 1983; Ungerstedt 1984; Stähle et al. 1991; Lindefors et al. 1989; Amberg and Lindefors (1989) and by Imperato and di Chiara 1984, 1985). In brain dialysis, a fine capillary fibre is implanted in a selected brain area. Low molecular weight compounds diffuse down their concentration gradients from the brain extracellular fluid into a physiological salt solution that flows through the capillary fibre at a constant rate. The fluid is collected and analyzed.

PROCEDURE

Several designs of dialysis probes have been used (Santiago and Westerink 1990; Kendrick 1991):

1. Horizontal probe

A straight tube (Vita Fiber, 3×50 Amicon) with an outer diameter of 0.34 mm and a molecular weight cut-off of 50 000 is used. The outer surface of the tube is porous and can easily be sealed by epoxy which is applied by passing the tube through a droplet of epoxy and then through a narrow hole corresponding to the outer diameter of the tube. The wall of the tube is sealed in this way except for the area where the dialysis is intended to take place. The length of this region can be varied from 2 to 8 mm depending upon which structure of the brain will be perfused. During the coating and all other handling of the tube it is supported by a thin tungsten or steel wire inserted into its lumen. One end of the tube is glued into a steel cannula (6 mm long, outer diameter 0.64 mm).

Male Sprague Dawley rats weighing 250–300 g are anesthetized with halothane and held in a stereotactic instrument. The animals are maintained under halothane anesthesia during the entire experiment.

Holes are drilled bilaterally (5.7 mm below and 1.5 mm in front of bregma) in the temporal bones after the temporal muscles have been retracted from the bones and folded away.

During the implantation, the cannula is held by the micromanipulator of the stereotactic instrument and the dialysis tube is passed horizontally through the brain through the holes drilled on both sides of the skull. A polyethylene tubing carrying the perfusion fluid is connected to the steel cannula. The perfusate is collected at the other end.

2. Loop probe

The probe is made of a flexible cellulosic tubing (Dow 50, outer diameter 0.25 mm). Both ends of the tube are inserted into 0.64 mm diameter steel tubes, one of which is bent in an angle. A very thin microsuture (0.1 mm in diameter) is inserted into the tube and positioned half between the steel tubes. Before implantation, the tube is moistened and bent in such a way that the two steel tubes are held closely together in the micromanipulator of the stereotactic instrument. A tungsten wire is inserted into the straight steel tube and passed down the lumen of the dialysis tube in order to stretch it and make it rigid enough to be implanted into the brain. The tube is implanted vertically and the steel cannulae are attached to the skull by dental cement. The tungsten wire is removed before starting the experiment. The cellulosic tube is flexible enough to withstand the bending at the lower end. The microsuture keeps the bend open.

Loop-shaped or U-shaped microdialysis probes have been used by several authors, e.g., Ichikawa and Meltzer (1990), Jordan et al. (1994), Westerink and Tuinte (1986), Auerbach et al. (1995).

3. Vertical probe

The probe is sealed at one end by epoxy. The other end is glued into a 0.64-mm diameter steel tube. A thin inner cannula made of a steel tube or a glass capillary carries the fluid to the bottom of the dialysis tube where it leaves the inner capillary and flows upwards and leaves the probe by a lateral tube. This vertical probe can also be coated with epoxy. It is especially suited for reaching ventral parts of the brain and performing dialysis in small nuclei of the brain.

A similar device has been described for continuous plasma sampling in freely moving rats by Chen and Steger (1993).

Most of the commercially available microdialysis probes are based on this principle.

4. Commercially available microdialysis probes

The microdialysis probes CMA/10, manufactured by Carnegie Medicine, Stockholm, Sweden, consist of a tubular membrane (polycarbonate; length: 3 mm; outside diameter: 0.50 mm; and inside diameter: 0.44 mm) glued to a cannula (outside diameter: 0.60 mm), and sealed with a glue at the tip (Stähle et al. 1991). The perfusion medium is carried to the dialyzing part of the probe by a thin cannula inside the probe. The medium leaves the inner cannula through two holes, flows back between the membrane and the inner cannula, and is collected at the outlet of the probe. The perfusion medium is delivered by means of a high precision micro-syringe pump.

This probe was used by several authors, e.g., Wood et al. (1988), Benveniste et al. (1989), Rollema et al. (1989), Scheller and Kolb (1991), Wang et al. (1993), Kreiss and Lucki (1995), Fink-Jensen et al. (1996).

CMA/11 probes were used by Boschi et al. (1995), Romero et al. (1996), Gobert et al. (1997).

Dialysis fibres with a semipermeable membrane AN 69-HF, Hospal-Dasco, Bologna, Italy, were used by de Boer et al. (1994), Rayevsky et al. (1995), Arborelius et al. (1996), Gainetdinov et al. (1996), Tanda et al. (1996).

EVALUATION

Samples of the dialysate are collected for different time intervals and analyzed for neurotransmitters. For the **evaluation of neuroleptics**, most authors measured dopamine, 3,4-dihydroxyphenyl acetic acid (DOPAC) and homovanillinic acid (HVA) by HPLC using appropriate detectors. See and Lynch (1996) analyzed dialysis samples for glutamate and GABA concentrations.

For the **evaluation of antidepressants** the concentrations of 5-hydroxytryptamine (5-HT), 5-hydroxy indole acetic acid (5-HIAA), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), or noradrenaline (NA) were measured in the effluent by HPLC. Wood et al. (1988), Egan et al. (1996) used 3-methoxytyramine accumulation as an index of dopamine release.

CRITICAL ASSESSMENT OF THE METHOD

The results obtained from brain dialysis depend on at least three variables: type of probe, post-implantation interval, and whether anesthetized or freely moving animals are used (Di Chiara 1990).

Several authors analyzed the diffusion processes underlying the microdialysis technique and described the limitations of the experiments (Jacobson et al. 1985; Amberg and Lindefors 1989; Beneviste et al. 1989; Scheller and Kolb 1991; Le Quellec et al. 1995).

As a matter of fact, brain microdialysis has been used for the evaluation of many drugs in various indications, such as:

- for **neuroleptics** by Ichikawa and Meltzer 1990; Meil and See 1994; Hernandez and Hoebel 1994; See et al. 1995; Schmidt and Fadayel 1995; Semba et al. 1995; Rayevsky et al. 1995; Fink-Jensen et al. 1996; See and Lynch 1996; Gainetdinov et al. 1996; Egan et al. 1996; Klitenick et al. 1996,
- for **antidepressants** by de Boer et al. 1994; Jordan et al. 1994; Arborelius et al. 1995; Ascher et al. 1995; Auerbach et al. 1995; de Boer 1995, 1996; Casanovas and Artigas 1996; Gobert et al. 1995; Ichikawa and Meltzer 1995; Kreiss and Lucki 1996; Petty et al. 1996; Potter 1996; Romero et al. 1995; Sharp et al. 1996; Tanda et al. 1996a,b,
- for studies in **Parkinson** models by Rollema et al. 1989; Parsons et al. 1991.

MODIFICATIONS OF THE METHOD

Ferrara et al. (1990) continuously monitored ethanol levels in the brain by microdialysis.

Hernandez and Hoebel (1994) performed simultaneous cortical, accumbens, and striatal microdialysis in freely moving rats.

Hegarty and Vogel (1995) assayed dopamine, DOPAC and HVA in the brain of rats after acute and chronic diazepam treatment and immobilization stress.

Casanovas and Artigas (1996) implanted microdialysis probes simultaneously in six different brain areas of rats (frontal cortex, dorsal striatum, ventral hippocampus, dorsal hippocampus, dorsal raphe nucleus, median raphe nucleus).

Beneviste et al. (1984) determined extracellular concentrations of glutamate and aspartate in rat hippoc-

ampus during transient cerebral ischemia monitored by intracerebral microdialysis.

Boschi et al. (1995) showed that microdialysis of small brain areas in mice is feasible using the smallest commercially available probes.

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E.5.1.14
Use of push-pull cannulae to determine the release of endogenous neurotransmitters

PURPOSE AND RATIONALE

Originally reported by Gaddum (1961), the push-pull cannula has become recognized and utilized as a powerful tool in conjunction with sufficiently sensitive assays to measure low levels of neuroregulator release in distinct brain areas *in vivo*. (Philippu 1984).

This method has been used for various purposes, e.g., to perfuse the ventricles of the brain with drugs, or to determine the release of labelled or endogenous compounds in the CSF (Bhattacharya and Feldberg 1958; Korf et al. 1976),

to perfuse distinct brain areas with drugs and to study their effects on functions of the central nervous system (Myers et al. 1976; Bhargava et al. 1978; Ruwe and Myers 1978),

to inject labelled monoamines or amino acids and to investigate the resting or induced release of radioactive compounds and their metabolites (Sulser et al. 1969; Strada and Sulser 1971; Kondo and Iwatsubo 1978),

to perfuse distinct brain areas with labelled transmitter precursors and to determine the patterns of release of the newly synthesized transmitters (Philippu et al. 1974; Chéramy et al. 1977; Nieoullon et al. 1977; Gauchy et al. 1980),

to perfuse distinct brain areas of anesthetized and conscious animals and to determine the release of endogenous neurotransmitters in the perfusate (Dluzen and Ramirez 1991).

PROCEDURE

The superfusion of the hypothalamus of the conscious, freely moving rabbit has been described by Philippu et al. (1981), Philippu (1984). Rabbits of both sexes are anesthetized with 40 mg/kg sodium pentobarbital i.p. Guide cannulae are mounted on a metal plate which is fixed on the skull with screws and dental cement. Some days after the operation, the guide cannulae are replaced with push-pull cannulae which are 4 mm longer than the guide cannulae, thus reaching the areas which are intended for superfusion. The push-pull cannulae are connected by tubing to two peristaltic pumps: one to push and another one to pull the fluid. The second pump is essential, because the superfusate is not directly collected from the side branch of the push-pull cannula, but from tubing which is connected to the side branch. The superfusate is automatically collected every 10 s in fraction collectors.

EVALUATION

The concentrations of neurotransmitters, e.g., epinephrine, norepinephrine, or dopamine are determined with appropriate analytical methods (Wolfensberger 1984) before and after stimulation.

MODIFICATIONS OF THE METHOD

Experiments in cats were described by Dietl et al. (1981), in rats by Tuomisto et al. (1983).

The cortical cup technique for collection of neurotransmitters has been described by Moroni and Pepeu (1984).

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E.5.1.15 Fos protein expression in brain

PURPOSE AND RATIONALE

The proto-oncogene c-fos encodes a 55 000 mol wt, 380 amino-acid phosphoprotein (FOS) which, after translation in the cytoplasm, re-enters the nucleus and binds to DNA (Morgan and Curran 1989). C-fos induction can occur as a consequence of synaptic activation. An increase in fos immunoreactivity is associated with an increased metabolic demand on a neuron, i.e., a marker for neurons that are metabolically activated. Intermediate early genes such as c-fos have been tentatively classified or linked to third messengers, whose function is to produce a long-term effect on the recipient neuron.

Acute administration of antipsychotics induces c-fos expression in several areas of the rat forebrain as was shown with immunocytochemical methods (Dragunow et al. 1990; Nguyen et al. 1992; Robertson and Fibiger 1992; MacGibbon et al. 1994). Fos protein is believed to act as an initiator of long-term cellular changes (neural plasticity) in response to a variety of extracellular stimuli, including drugs (Graybiel et al. 1990; Rogue and Vicendon 1992). Typical (e.g. haloperidol) and atypical (e.g. clozapine) neuroleptic drugs have different antipsychotic effects and side-effects. A differential FOS-protein induction in rat forebrain regions after haloperidol and clozapine treatment was found (Deutch et al. 1992; Fibiger 1994; Fink-Jensen and Kristensen 1994; Merchant et al. 1994; Sebens et al. 1995). The induction pattern of Fos-like immunoreactivity in the forebrain could serve as predictor of atypical antipsychotic drug activity (Robertson et al. 1994).

PROCEDURE

Groups of 4–6 male Wistar rats weighing 350–450 g are injected subcutaneously with saline (control) or with various doses of the standard drugs or compounds with putative antipsychotic activity. After two hours, the animals are deeply anesthetized by intraperitoneal injection of 100 mg/kg pentobarbital and perfused with 200 ml saline followed by 200 ml of 4% paraformaldehyde in phosphate buffer solution (PBS). Each brain is removed immediately after perfusion and placed in fresh fixative for at least 12 h.

After the postfixative period, 30-µm sections are cut from each brain using a vibratome. Several antisera to detect Fos can be used, such as a sheep polyclonal antibody directed against residues 2 to 16 of the N-terminal region of the Fos molecule, or a polyclonal antiserum raised in rabbits against Fos peptide (4–17 amino acids of human Fos).

Sections are washed 3 times with 0.02 mM PBS and then incubated in PBS containing 0.3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections are then washed 3 times in PBS and incubated in PBS containing 0.3% Triton X-100, 0.02% azide and Fos primary antisera (diluted 1 : 200) for 48 h. The sections are then washed 3 times with PBS and incubated with a biotinylated rabbit antisheep secondary antibody (diluted 1 : 200) for 1 h. The sections are washed three times with PBS and incubated for 1 h with PBS containing 0.3% Triton X-100 and 0.5% avidin-biotinylated horseradish peroxidase complex. After 3 washes in PBS the sections are rinsed in 0.1 M acetate buffer, pH 6.0. Fos immunoreactivity is revealed by placing the sections in a solution containing 0.05% 3,3'-diaminobenzidine, 0.2% ammonium nickel sulfate and 0.01% H_2O_2. The reaction is terminated with a washing in acetate buffer. The sections are mounted on chrome-alum-coated slides, dehydrated and prepared for microscopic observation.

Drug induced changes in Fos-like immunoreactivity are quantified by counting the number of immunoreactive nuclei in the medial prefrontal cortex, nucleus accumbens, medial and dorsolateral striatum and the lateral septal nucleus. The number of Fos-positive nuclei are counted with a 550×550 µm grid placed over each of these regions with a 100× magnification.

Typical and atypical antipsychotics can be classified on the basis of difference between Fos-like immunoreactivity in the nucleus accumbens and lateral striatum. For this purpose, the data are corrected for the effects which are produced by the injection procedure itself. The injection corrected value for the dorsolateral striatum is subtracted from the corresponding accumbal value for each drug dose.

This manipulation yields a value termed the atypical index, i.e. number of Fos-positive neurons in the nucleus accumbens minus the number in the lateral striatum = atypical index. A negative index indicates the probability of side-effects, like extrapyramidal syndrome, exerted by the typical neuroleptics, a positive value to be devoid of it.

EVALUATION

A one-way analysis of variance is performed on the cell count data for each dose and the corresponding vehicle control. If the analysis of variance is significant, multiple comparisons are performed by using the Newman-Keuls test.

MODIFICATIONS OF THE METHOD

Graybiel et al. (1990) reported a drug-specific activation of c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum by amphetamine and cocaine.

Deutch et al. (1991) found that stress selectively increases Fos protein in dopamine neurons innervating the prefrontal cortex.

Gogusev et al. (1993) described modulation of C-fos and other proto-oncogene expression by phorbol diester in a human histiocytosis DEL cell.

Deutch et al. (1995) studied the induction of Fos protein in the thalamic paraventricular nucleus as locus of antipsychotic drug action.

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E.5.1.16 Neurotensin

E.5.1.16.1 General considerations on neurotensin and neurotensin receptors

Neurotensin is a 13 amino acid peptide originally isolated from calf hypothalamus (Carraway and Leeman 1973). It is secreted by peripheral and neuronal tissues and produces numerous pharmacological effects in animals suggesting **analgesic** (Coguerel et al. 1988; Clineschmidt and McGuffin 1977; Smith et al. 1997), **cardiovascular** (Carraway and Leeman 1973; Schaeffer et al. 1998; Seagard et al. 2000), **hypothermic** (Bissette

et al. 1976; Benmoussa et al. 1996; Tyler-McMahon et al. 2000), and **antipsychotic** (Nemeroff 1986; Sarhan et al. 1997; Feifel et al. 1999; Kinkead et al. 1999; Cusack et al. 2000) actions. Neurotensin is even considered to be an endogenous neuroleptic (Ervin and Nemeroff 1988; Gully et al. 1995). Radke et al. (1998) studied synthesis and efflux of neurotensin in different brain areas after acute and chronic administration of typical and atypical antipsychotic drugs.

Neurotensin affects **gastrointestinal functions**, such as stimulating growth of various gastrointestinal tissues (Feurle et al. 1987), modulation of pre- and postprandial intestinal motility (Pellissier et al. 1996), inhibiting gastric acid secretion (Zhang et al. 1989a), inducing contractile responses in intestinal smooth muscle (Unno et al. 1999), maintaining gastric mucosal blood flow during cold water restraint (Zhang et al. 1989b; Xing et al. 1998).

Neurotensin acts as a **growth factor** on a variety of normal and cancer cells (Wang et al. 2000).

Like other neuropeptides, neurotensin is synthesized as part of a larger precursor which also contains neuromedin N, a six amino acid neurotensin-like peptide belonging to the gastrin-releasing peptide/bombesin family (see J.3.1.8).

Several peptidic and non-peptidic neurotensin agonists and antagonists have been synthesized and analyzed in pharmacological tests as potential drugs mainly in psychopharmacology (Gully et al. 1995, 1996, 1997; Azzi et al. 1996; Castagliuolo et al. 1996; Chapman and See 1996; Mule et al. 1996; Hong et al. 1997; Johnson et al. 1997; Sarhan et al. 1997; Betancur et al. 1998; Gudasheva et al. 1998; Schaeffer at al 1998). Furthermore, inhibitors of neurotensin-degrading enzymes were described (Bourdel et al. 1996).

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E.5.1.16.2 Neurotensin receptor binding

PURPOSE AND RATIONALE

Neurotensin interacts with two cloned receptors that were originally differentiated on the basis of their affinity to the antihistaminic drug levocabastine (Schotte et al. 1986). The high sensitive, levocabastine-insensitive rat neurotensin receptor (*NTR1*) was cloned first (Tanaka et al. 1990) and shown to mediate a number of peripheral and central neurotensin responses, including the neuroleptic-like effects of the peptide (Labbé-Jullié et al. 1994). The human NTR1 has been cloned from the colonic adenocarcinoma cell line HT29 (Vita et al. 1993) and shown to consist of a 416 amino acid protein that shares 84% homology with rat NTR1. A second human NTR1 receptor differing only in one amino acid has been cloned from substantia nigra by Watson et al. (1993).

The lower-affinity, levocabastine-sensitive neurotensin receptor (*NTR2*) was cloned by Chalon et al. (1996), Mazella et al. (1996) and characterized by Yamada et al. (1998). Studies by Dubuc et al. (1999) indicate that NTR2 mediates neurotensin-induced analgesia.

A third neurotensin receptor (*NTR3*) was cloned from a human brain cDNA library (Mazella et al. 1998). It is identical with sortilin, a receptor-like protein, cloned from human brain (Petersen et al. 1997, 1999). The NT3/gp95/sortilin protein is a transmembrane neuropeptide receptor which does not belong to the superfamily of G-protein-coupled receptors.

Gully et al. (1997) described a binding assay for the neurotensin1 receptor.

PROCEDURE

Cell culture

CHO cells transfected with cDNA of the human neurotensin receptor cloned from HT 29 cells (h-NTR1-CHO cells) are cultured at 37 °C in modified Eagles medium without nucleosides, containing 10% fetal calf serum, 4 mM glutamine and 300 µg/ml geneticin (G418), in a humidified incubator under 5% CO_2 in O_2. The colonic adenocarcinoma HT 29 cell line (American Type Culture Collection, Rockville, MD) is cultured under similar conditions in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal calf serum, 4 mM glutamine, 200 IU/ml penicillin and 200 mg/ml streptomycin. One week after seeding, confluent monolayer cultures are washed three times with 3 ml PBS and harvested by enzymatic dissociation with trypsin. After dilution with PBS, cells are resuspended in the same culture medium at a density of 5×10^4 cells/ml and are plated into 35-mm-diameter, fibronectin-coated Petri culture dishes.

Membrane homogenate preparation and binding assay

Whole brains of male Sprague-Dawley rats albino guinea pigs or cell pellets are homogenized in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) for 30 s, using a Polytron homogenizer (setting 5). After 20 min centrifugation at 30000*g*, the pellet is washed, centrifuged again under the same conditions, resuspended in a storage buffer containing 50 mM Tris-HCl

(pH 7.4), 1 mM EDTA, 0.1% BSA, 40 mg/liter bacitracin, 1 mM 1,10-orthophenanthroline and 5 mM dithiothreitol and stored as aliquots in liquid nitrogen until used.

Aliquots of membranes (10, 50, 300 and 500 µg of protein for h-NTR1-CHO cells, HT 29 cells, rat and guinea pig brain, respectively) are incubated for 20 min at 20 °C in the incubation buffer (0.5 ml final volume) containing appropriate concentrations of [^{125}I-Tyr^3]neurotensin (25–100 pM) and unlabeled drugs. After incubation, the assay medium is diluted with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) supplemented with 0.1% BSA and 1 mM EDTA, and the mixture is rapidly filtered under reduced vacuum through Whatman GF/B glass fibre filters that have been pretreated with 0.1% polyethyleneimine. The filters are washed under the same conditions three times and radioactivity is measured. Nonspecific binding is determined in the presence of 1 µM unlabeled neurotensin. All experiments are performed in triplicate, and data are expressed as the mean ±SEM of at least three separate determinations.

EVALUATION

The IC_{50} is the value of ligand that inhibits 50% of the specific binding and is determined using an iterative nonlinear regression program (Munson and Rodbard 1980).

MODIFICATIONS OF THE METHOD

Cusack et al. (1995) studied species selectivity of neurotensin analogs at the rat and two human NTR1 receptors.

Le et al. (1997) cloned the human neurotensin receptor gene and determined the structure.

Labbé-Jullié et al. (1998) attempted to identify residues in the rat NTR1 that are involved in binding of a nonpeptide neurotensin antagonist.

Souazé et al. (1997), Najimi et al. (1998) studied the effects of a neurotensin agonist and showed in human colonic adenocarcinoma HT 29 cells after short incubation an increase, after prolonged exposure a decrease of mRNA levels and in the human neuroblastoma cell line CHP 212 a high affinity neurotensin receptor gene activation.

Ovigne et al. (1998) described a monoclonal antibody specific for the human NTR1.

Nouel et al. (1999) found that both NT2 and NT3 neurotensin receptor subtypes were expressed by cortical glial cells in culture.

Cusack et al. (2000) developed a neurotensin analog, NT34, that can distinguish between rat and human neurotensin receptors, and exhibits more than a 100-fold difference in binding affinities.

Neuromedin N, a peptide belonging to the gastrin-releasing peptide/bombesin family (see J.3.1.7) shows a high affinity to brain neurotensin receptors and is rapidly inactivated by brain synaptic peptidases (Checler et al. 1990).

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E.5.2 Behavioral tests

E.5.2.1 Golden hamster test

PURPOSE AND RATIONALE

"Innate behavior" of many species including man has been described by Lorenz (1943, 1966). The "Golden-hamster-test" (Ther, Vogel and Werner 1959) uses the innate behavior of this species (*Mesocricetus auratus*) for differentiation between neuroleptic and sedative – hypnotic activity. The aggressive behavior of male golden hamsters is suppressed by neuroleptics in doses which do not impair motor function.

PROCEDURE

Ten to 20 male golden hamsters with an average weight of 60 g are crowded together in Makrolon$^{(R)}$ cages for at least 2 weeks. During this time the animals develop a characteristic fighting behavior. For the test single animals are placed into glass jars of 2 liters. In this situation the hamsters assume a squatting and resting position during the day. If the animals are touched with a stick or a forceps they wake up from their daytime sleep and arouse immediately from the resting position. If one tries to hold the hamster with a blunted forceps, a characteristic behavior is elicited: The hamster throws himself onto his back, tries to bite and to push the forceps away with his legs, and utters angry shrieks. Touching the animals is repeated up to 6 times followed by punching with the forceps. Only animals responding to the stimulus with all three defense reactions (turning, vocalizing, biting) are included into the test.

The test compounds are applied either subcutaneously, intraperitoneally, or orally. Six animals are used for each dose.

EVALUATION

The stimuli are applied every 20 min for 3 h. The number of stimuli until response is recorded. Furthermore, the suppression of the defense reactions (turning, biting and vocalizing) is evaluated. An animal is regarded to be completely "tamed", if all defense reactions are suppressed even after punching with the forceps at least once during the test period.

After each stimulation the "tamed" animal is placed on an inclined board with 20 degree inclination. Normal hamsters and hamsters tamed by neuroleptics are able to support themselves or to climb on the board. Impaired motor function causes sliding down. This experiment is repeated three times after each testing of the defense reactions. An animal's coordination is considered to be disturbed if it falls three times during two tests of the experiments.

For each dose the number of tamed hamsters and the number of animals with impaired motor function is recorded. Using different doses, ED_{50} values can be calculated for the taming effect and for impairment of motor function.

The ED_{50} values of taming were 1.5 mg/kg for chlorpromazine s.c. and 0.2 mg/kg for reserpine s.c. Much higher doses (10 times of chlorpromazine and 5 times of reserpine) did not elicit motor disturbances. On the

contrary, while ED_{50} values of 10 mg/kg phenobarbital s.c. and 180 mg/kg meprobamate p.o. for the taming effect were found, these doses already caused severe motor disturbances. The taming dose of diazepam was 10 mg/kg p.o. which already showed some muscle relaxing activity. The term "neuroleptic width" indicates the ratio between the ED_{50} for taming and the ED_{50} for motor disturbances. Only for neuroleptic drugs are ratios found between 1 : 5 and 1 : 30.

CRITICAL ASSESSMENT OF THE METHOD

The method has the advantage that neuroleptics can easily be differentiated from sedative and hypnotic drugs. Anxiolytics with pronounced muscle relaxing activity also show no significant differences between taming and impaired motor function. Moreover, the method has the advantage that no training of the animals and no expensive apparatus is needed.

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E.5.2.2 Influence on behavior of the cotton rat

PURPOSE AND RATIONALE

The "cotton-rat test" is another attempt to use the innate behavior as described for several animal species by Lorenz (1943, 1966) for the differentiation of psychotropic drugs (Vogel and Ther 1960). The cotton rat (*Sigmodon hispidus*) is a very shy animal which conceals himself at any time. This innate flight reflex is suppressed by centrally active drugs. Simultaneous evaluation of motor function allows the differentiation between neuroleptic and sedative drugs.

PROCEDURE

Cotton rats are bred in cages equipped with a clay cylinder of 20 cm length and 10 cm diameter. This cylinder is used by the animals for hiding, sleeping and breeding. Moreover, the animals which bite easily can be transported from one cage to another just by closing the cylinder on both ends. For the test young animals with a body weight of 40 g are used. Young animals are as shy as old ones but less vicious. Nevertheless, leather gloves have to be used for handling of cotton rats. Normal cages ($25 \times 30 \times 20$ cm) with a wire lid are used. A tunnel of sheet metal (half of a cylinder) 20 cm long and 7 cm high is placed into the cage. The cotton rats hide immediately in this tunnel. If the tunnel is lifted and placed on another site of the cage, the cotton rats immediately hide again.

Three rats are placed in one cage and tested for their behavior. Selective shaving of the fur enables the observer to recognize each animal. If the rats behave as described, they are then treated with the test compound subcutaneously or orally. At least 6 animals divided in two cages are used for each dose of test compound or standard. Fifteen min after application of the drug the test period of three h is started. The tunnel is lifted and placed to another site. If the animals do not show the immediate flight reflex an airstream of short duration is blown through the wire lid. If the animal still does not respond with the flight reflex it is considered to be positively influenced. Afterwards, the animal is placed on an inclined board with 35 degree of inclination and tested for disturbance of motor coordination. A normal animal is able to climb upwards. If coordination is disturbed the rat slides down.

EVALUATION

The test procedure is repeated every 15 min over a period of 3 h. The animals which show at least one suppression of the flight reflex during the test period are counted as well as those who slide down on the inclined board. Using different doses ED_{50} values are calculated for both parameters. The ratio between these two ED_{50} values is regarded as "neuroleptic width" which is 1 : 20 for chlorpromazine and 1 : 30 for reserpine whereas ratios of 1 : 2 for phenobarbital and 1 : 1.5 for meprobamate indicate the absence of neuroleptic activity.

CRITICAL ASSESSMENT OF THE METHOD

The method allows the differentiation of drugs with neuroleptic activity against other centrally active drugs. No training of the animals and no expensive equipment are necessary.

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E.5.2.3 Artificial hibernation in rats

PURPOSE AND RATIONALE

Giaja (1938, 1940, 1953, 1954) studied the effects of reduced oxygen tension and cold environment on rats. The animals were placed in hermetically closed glass vessels which were submerged in ice-water. Due to the respiratory activity, the oxygen tension diminishes and the carbon dioxide content increases. Under the influence of cooling and of hypoxic hypercapnia, the rectal temperature falls to 15 °C and the animal is completely anesthetized and immobilized. The rat can survive in this poikilothermic state for more than twenty hours. Complete recovery occurs after warming up. This kind of artificial hibernation was augmented by chlorpromazine (Courvoisier et al. 1953; Giaja and Markovic-Giaja 1954). Vogel (1959), Ther et al. (1959, 1963) used these observations for evaluation of neuroleptics and opioid analgesics.

PROCEDURE

Male Wistar rats weighing 100–150 g are deprived of food with free access to tap water overnight. The test compounds are injected subcutaneously 15 min prior to the start of the experiment. First, the rats are placed in ice-cold water to which surfactant is added in order to remove the air from the fur for 2 min. Then, the animals are placed into hermetically closed glass vessels of 750 ml volume which are placed into a refrigerator at 2 °C temperature. During the following hour, the vessels are opened every 10 min for exactly 10 s allowing some exchange of air and reducing the carbon dioxide accumulation. At each time, animals are removed from the glass vessel and observed for signs of artificial hibernation which are not shown by control animals under these conditions. Treated animals, lying on the side, are placed on the back and further examined. An animal is considered positive, when it remains on the back, even if the extremities are stretched out. In this state, cardiac and respiration frequency are reduced and the rectal temperature has fallen to 12–15 °C. The rigor of the musculature allows only slow movements of the extremities. The animals recover completely within a few hours if they are brought to their home cages at room temperature. Artificial hibernation is induced dose-dependent by neuroleptics of the phenothiazine type and by some opioid analgesics like meperidine and methadone. In contrast, morphine shows only slight activity.

EVALUATION

Various doses are applied to groups of 10 animals. Percentage of positive animals is calculated for each group and ED_{50} values with confidence limits are estimated according to Litchfield and Wilcoxon.

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E.5.2.4 Catalepsy in rodents

PURPOSE AND RATIONALE

Catalepsy in rats is defined as a failure to correct an externally imposed, unusual posture over a prolonged period of time. Neuroleptics which have an inhibitory action on the nigrostriatal dopamine system induce catalepsy (Costall and Naylor 1974; Chermat and Simon 1975; Sanberg 1980) while neuroleptics with little or no nigrostriatal blockade produce relatively little or no cataleptic behavior (Honma and Fukushima 1976). Furthermore, cataleptic symptoms in rodents have been compared to the Parkinson-like extrapyramidal side effects seen clinically with administration of antipsychotic drugs (Duvoisin 1976).

PROCEDURE

Groups of 6 male Sprague-Dawley or Wistar rats with a body weight between 120 and 250 g are used. They are dosed intraperitoneally with the test drug or the standard. Then, they are placed individually into translucent plastic boxes with a wooden dowel mounted horizontally 10 cm from the floor and 4 cm from one

end of the box. The floor of the box is covered with approximately 2 cm of bedding material. White noise is presented during the test. The animals are allowed to adapt to the box for 2 min. Then, each animal is grasped gently around the shoulders and under the forepaws and placed carefully on the dowel. The amount of time spent with at least one forepaw on the bar is determined. When the animal removes its paws, the time is recorded and the rat is repositioned on the bar. Three trials are conducted for each animal at 30, 60, 120 and 360 min.

EVALUATION

An animal is considered to be cataleptic if it remains on the bar for 60 s. Percentage of cataleptic animals is calculated. For dose-response curves, the test is repeated with various doses and more animals. ED_{50} values can be calculated. A dose of 1 mg/kg i.p. of haloperidol was found to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The phenomenon of catalepsy can be used for measuring the efficacy and the potential side effects of neuroleptics.

MODIFICATIONS OF THE METHOD

Extrapyramidal syndromes after treatment with typical and atypical neuroleptics were measured in nonhuman primates (Cebus monkeys) by Casey (1989, 1991, 1993), Gerlach and Casey (1990).

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E.5.2.5 Pole climb avoidance in rats

PURPOSE AND RATIONALE

The pole-climb avoidance paradigm is an avoidance-escape procedure used to separate neuroleptics from sedatives and anxiolytics. Whereas sedative compounds suppress both avoidance and escape responding at approximately the same doses, neuroleptic drugs reduce avoidance responding at lower doses than those affecting escape responding (Cook and Catania 1964).

PROCEDURE

Male rats of the Long-Evans strain with a starting body weight of 250 g are used. The training and testing of the rats is conducted in a $25 \times 25 \times 40$ cm chamber that is enclosed in a dimly lit, sound-attenuating box. Scrambled shock is delivered to the grid floor of the chamber. A 2.8-kHz speaker and a 28-V light are situated on top of the chamber. A smooth stainless-steel pole, 2.5 cm in diameter, is suspended by a counterbalance weight through a hole in the upper center of the chamber. A microswitch is activated when the pole is pulled down 3 mm by a weight greater than 200 g. A response is recorded when a rat jumps on the pole and activates the microswitch. The rat can not hold the pole down while standing on the grid floor because of the counterbalance tension and can not remain on the pole any length of time because of its smooth surface. The activation of the light and the speaker together are used as the conditioning stimulus. The conditioning stimulus is presented alone for 4 s and then is coincident with the unconditioned stimulus, a scrambled shock delivered to the grid floor, for 26 s. The shock current is maintained at 1.5 mA. A pole climb response during the conditioned stimulus period terminates the conditioned stimulus and the subsequent conditioned and unconditioned stimuli. This is considered an avoidance response. A response during the time when both the

conditioned and unconditioned stimuli are present terminates both stimuli and is considered an escape response. Test sessions consist of 25 trials or 60 min, whichever comes first. There is a minimum intertrial interval of 90 s. Any time remaining in the 30 s allotted to make the pole climb is added to the 90 s intertrial interval. Responses during this time have no scheduled consequences; however, rats having greater than 10 intertrial interval responses should not be used in the experiment. Before testing experimental compounds, rats are required to make at least 80% avoidance responses without any escape failures.

EVALUATION

Data are expressed in terms of the number of avoidance and escape failures relative to the respective vehicle control data. ED_{50} values can be calculated using different doses.

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Locke KW, Dunn RW, Hubbard JW, Vanselous CL, Cornfeldt M, Fielding S, Strupczewski JT, (1990) HP 818:A centrally acting analgesic with neuroleptic properties. Drug Dev Res 19:239–256

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E.5.2.6 Foot-shock induced aggression

PURPOSE AND RATIONALE

The test as described by Tedeschi et al. (1959) using mice which fight after foot-shock induced stimulation is useful to detect neuroleptics but also shows positive effects with anxiolytics and other centrally effective drugs. The method has been used by several authors to test drugs with neuroleptic activity. The test is described in Sect. E.2.3.1.

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E.5.2.7 Brain self stimulation

PURPOSE AND RATIONALE

In several species, electrical stimulation of selected brain loci produces effects which are positively reinforcing and pleasurable (Olds and Milner 1954; Olds 1961, 1972). Most of the data available have been obtained from experiments using rats with electrodes chronically implanted in the median forebrain bundle at the level of hypothalamus. Minute electrical pulses sustain a variety of operant behaviors such as lever pressing. Neuroleptics have been shown to be potent blockers of self stimulation (Broekkamp and Van Rossum 1975; Koob et al. 1978; Gallistel and Freyd 1987). Conversely, compounds that facilitate catecholaminergic transmission such as d-amphetamine and methylphenidate, will increase responding for such stimulation.

PROCEDURE

Male Wistar rats (350–400 g) are anesthetized with 50 mg/kg pentobarbital i.p. and their heads placed on a level plane in a Kopf stereotactic instrument. A midline incision is made in the scalp and the skin held out of the way by muscle retractors. A small hole is drilled in the scull with a dental burr at the point indicated by the stereotactic instrument for the structure it is desired to stimulate. Using bregma as a reference point, the electrode (Plastic Products # MS303/1) is aimed at the medium forebrain bundle according to the atlas of Paxinos and Watson (1986), using the coordinates of AP = –0.8 mm, Lat = +2.8 mm, and DV = –7.2 mm below dura. The assembly is then permanently affixed to the scull using stainless steel screws and bone cement.

After a minimum of 10 days for recovery, the animals are trained to bar press for electrical stimulation on a continuous reinforcement schedule in a standard operant box outfitted with a single lever. The reward stimulus is a train of biphasic square-wave pulses generated by a Haer stimulator (Pulsar 4i). The parameters are set at a pulse duration of 0.5 ms with 2.5 ms between each pulse pair. The train of pulses may vary between 16 and 30/s, and the intensity of the pulses that are delivered range from 0.1 to 0.5 mA using the lowest setting that will sustain maximal responding. After consistent baseline responding is obtained for 5 consecutive 30 min sessions, the animals are ready for testing with standard agents. Compounds are administered 60 min prior to testing. All data are collected on both cumulative recorders and counters.

EVALUATION

The number of drug responses are compared to the number of responses made during each animal's 30 min control session on the preceding day, which is considered to be equal to 100%. Testing various doses, ED_{50} values with 95% confidence limits can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

Since there is sufficient evidence that self-stimulation behavior is maintained by catecholamines the method gives indirectly insight into the catecholaminergic facilitating or blocking properties of a compound. Active neuroleptic drugs inhibit the self-stimulation behavior in very small doses. The relative potency observed in this test of clinically efficacious drugs parallels their potency in the treatment of schizophrenia.

MODIFICATIONS OF THE METHOD

Reinforcing brain stimulation by electrodes placed in the medial forebrain bundle of rats is decreased after lesion of the internal capsule in the region of the diencephalic-telencephalic border. This decrement in rewarding processing can be reversed by antidepressant drugs (Cornfeldt et al. 1982).

Depoortere et al. (1996) used electrical self stimulation of the ventral tegmental area to study the behavioral effects of a putative dopamine D_3 agonist in the rat.

Anderson et al. (1995) examined the interaction of aversive and rewarding stimuli in self stimulating rats in terms of duration and direction. The rats were implanted with two moveable electrodes, one in a region supporting self-stimulation (the ventral tegmental area) and another in a region supporting escape (the nucleus reticularis gigantocellularis).

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E.5.3 Tests based on the mechanism of action

E.5.3.1 Amphetamine group toxicity

PURPOSE AND RATIONALE

It is well known that aggregation of mice in small cages greatly enhances the toxicity of amphetamine. The death rate can be reduced by pretreatment with neuroleptics.

This phenomenon is generally accepted as an indicator of neuroleptic activity. The increased toxicity results from increased behavioral activation due to aggregation inducing an increase of circulating catecholamines. The mechanism can be understood by the fact that amphetamine is an indirectly acting sympathomimetic amine that exerts its effects primarily by releasing norepinephrine from storage sites in the sympathetic nerves. After administration of high doses of amphetamine, mice exhibit an elevated motor activity which is highly increased by aggregation. This increased behavioral activation is followed by death within 24 h in 80–100% of control animals. Neuroleptics reduce this death rate. In contrast, non-neuroleptic sympatholytics and psychosedative agents like the barbiturates do not produce a dose-related protection. Moreover, anxiolytic agents like benzodiazepines are also found to be ineffective in the prevention of amphetamine group toxicity.

PROCEDURE

Ten male mice of the NMRI-strain are used for each group. They are dosed with the test compound or the standard either orally or intraperitoneally and all placed in glass jars of 18 cm diameter. Untreated animals serve as controls. The test has to be performed at room temperature of 24 °C. Thirty min after i.p. or 1 h after oral administration the mice receive 20 mg/kg d-amphetamine subcutaneously. The mortality is assessed 1, 4 and 24 h after dosing.

EVALUATION

The mortality of amphetamine only treated animals is at least 80%. If less than 80% die due to low ambient temperature the test has to be repeated. The estimation of ED_{50} values for protection and their confidence limits are calculated by probit analysis of the data using the number of dosed vs. the number of surviving animals. Doses of 10 mg/kg chlorpromazine p.o. and 1 mg/kg haloperidol have been found to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The amphetamine group toxicity test has been used by many investigators and has been found to be a reliable method for detecting neuroleptic activity.

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E.5.3.2 Inhibition of amphetamine stereotypy in rats

PURPOSE AND RATIONALE

Amphetamine is an indirect acting sympathomimetic agent which releases catecholamines from its neuronal storage pools. In rats the drug induces a characteristic stereotypic behavior. This behavior can be prevented by neuroleptic agents.

PROCEDURE

Groups of 6 Wistar rats with a body weight between 120 and 200 g are used. They are injected simultaneously with d-amphetamine (10 mg/kg s.c.) and the test compound intraperitoneally and than placed individually in stainless-steel cages ($40 \times 20 \times 18$ cm). The control groups receive d-amphetamine and vehicle. Stereotypic behavior is characterized by continuous sniffing, licking or chewing and compulsive gnawing. The animals are observed 60 min after drug administration. An animal is considered to be protected, if the stereotypic behavior is reduced or abolished.

EVALUATION

The percent effectiveness of a drug is determined by the number of animals protected in each group. A dose response is obtained by using 10 animals per group at various doses. ED_{50} values can be calculated. The standard neuroleptic drugs have the following ED_{50} values: chlorpromazine 1.75 mg/kg i.p and haloperidol 0.2 mg/kg i.p.

CRITICAL ASSESSMENT OF THE METHOD

Inhibition of amphetamine-induced stereotypies in rats can be regarded as a simple method to detect neuroleptic activity. However, this may reflect the effects in the corpus striatum which are thought to be responsible for the Parkinsonism-like side effects of neuroleptics.

MODIFICATIONS OF THE METHOD

Ljungberg and Ungerstedt (1985) described a rapid and simple behavioral screening method for simultaneous assessment of limbic and striatal blocking effects of neuroleptic drugs. A low dose of 2 mg/kg d-amphetamine i.p. induces both increased locomotion, thought to reflect an increased dopamine transmission in the nucleus accumbens, and weak stereotypies, thought to reflect an increased dopamine transmission in the neostriatum. The behavior is measured in a combined open field apparatus with holes on the bottom to measure nose-pocking and registration of time spent in the corners. Neuroleptics with less propensity to induce unwanted extrapyramidal side effects can be differenti-

ated from classical drugs with more extrapyramidal adverse reactions.

Machiyama (1992) recommended chronic methylamphetamine intoxication in **Japanese monkeys** (*Macaca fuscata*) as a model of schizophrenia in animals.

Ellenbroek (1991) described the ethological analysis of **Java monkeys** (*Macaca fascicularis*) in a social setting as an animal model for schizophrenia.

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E.5.3.3 Inhibition of apomorphine climbing in mice

PURPOSE AND RATIONALE

Administration of apomorphine to mice results in a peculiar climbing behavior characterized initially by rearing and then full-climbing activity, predominantly mediated by the mesolimbic dopamine system (Costall et al. 1978). The ability of a drug to antagonize apomorphine-induced climbing behavior in the mouse has been correlated with neuroleptic potential (Protais et al. 1976; Costall et al. 1978).

PROCEDURE

Groups of 10 male mice (20–22 g) are treated i.p. or orally with the test substance or the vehicle and placed individually in wire-mesh stick cages. Thirty min afterwards, they are injected s.c. with 3 mg/kg apomorphine. Ten, 20 and 30 min after apomorphine administration, they are observed for climbing behavior and scored as follows:

0 = four paws on the floor,
1 = forefeet holding the vertical bars,
2 = four feet holding the bars.

EVALUATION

The average values of the drug-treated animals are compared with those of the controls, the decrease is expressed as percent. The ED_{50}-values and confidence limits are calculated by probit analysis. Three dose levels are used for each compound and the standard with a minimum of 10 animals per dose level.

CRITICAL ASSESSMENT OF THE TEST

Similar to the enhancement of compulsive gnawing of mice after apomorphine by antidepressant drugs, the suppression of climbing behavior of mice after apomorphine can be used for testing neuroleptic drugs. The test has been modified by various authors.

In contrast to other strains of mice, apomorphine climbing is not induced in DBA2 mice unless subchronic manipulations of brain dopamine transmission are performed (Duterte-Boucher and Costentin 1989).

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E.5.3.4 Inhibition of apomorphine stereotypy in rats

PURPOSE AND RATIONALE

Apomorphine induces a stereotyped behavior in rats, characterized by licking, sniffing and gnawing in a repetitive, compulsive manner, which is an indication of striatal dopaminergic stimulation (Anden et al. 1967; Ernst 1967; Costall and Naylor 1973). Compounds which prevent apomorphine-induced stereotypy antagonize dopamine receptors in the nigrostriatal system (Ljungberg and Ungerstedt 1978; Tarsy and Baldessarini 1974). Furthermore, antagonism of this behavior is predictive of propensity for the development of extrapyramidal side effects and tardive dyskinesias (Klawans and Rubivits 1972; Tarsy and Baldessarini 1974; Christensen et al. 1976; Clow et al. 1980).

PROCEDURE

For screening, groups of 6 male Wistar rats with a body weight between 120 and 200 g are used. The test drug or the standard are administered i.p. 60 min prior apomorphine dosage. Apomorphine HCl is injected s.c. at a dose of 1.5 mg/kg. The animals are placed in individual plastic cages. A 10 s observation period is used to measure the presence of stereotypic activity such as sniffing, licking and chewing 10 min after apomorphine administration. An animal is considered protected if this behavior is reduced or abolished.

EVALUATION

The percent effectiveness of a drug is determined by the number of animals protected in each group. With a group size of 10 animals dose response curves are obtained and ED_{50} values calculated. ED_{50} values were found to be 0.2 mg/kg s.c. for haloperidol and 5.0 mg/kg for chlorpromazine, whereas clozapine was ineffective even at high doses.

MODIFICATIONS OF THE METHODS

Puech et al. (1978) studied the effects of several neuroleptic drugs on hyperactivity induced by a low dose of apomorphine in mice.

Apomorphine induces stereotypic behavior in a variety of species including pigeons. The symptoms in pigeons are manifested as pecking against the wall of the cage or on the floor. Aksas et al. (1984) described a method registering the pecking after apomorphine by a microphone, amplification through a pulse preamplifier and registration with a polygraph. The effect of apomorphine was dose-dependent decreased by yohimbine and neuroleptics.

Stereotyped behavior in guinea pigs induced by apomorphine or amphetamine consisting in continuous gnawing and sniffing of the cage floor was described by Klawans and Rubovits (1972) and used as an experimental model of tardive dyskinesia.

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E.5.3.5 Yawning/penile erection syndrome in rats

PURPOSE AND RATIONALE

Yawning is a phylogenetically old, stereotyped event that occurs alone or associated with stretching and/or penile erection in humans and in animals from reptiles to birds and mammals under different conditions (Argiolas and Melis 1998). The yawning-penile erection syndrome can be induced in rats by apomorphine and other dopamine autoreceptor stimulants (Ståhle and Ungerstedt 1983; Gower et al. 1984) and can be antagonized by haloperidol and other dopamine antagonists. Antagonism against this syndrome can be regarded as indication of antipsychotic activity (Furukawa 1996).

Besides the **dopaminergic** system in this behavior (Mogilnicka and Klimek 1977; Baraldi et al. 1979; Benassi-Benelli et al. 1979; Nickolson and Berendsen 1980; Gower et al. 1984, 1986; Dourish et al. 1985; Doherty and Wisler 1994; Kurashima et al. 1995; Bristow et al. 1996; Fujikawa et al. 1996a; Asencio et al. 1999) also the **serotoninergic** (Baraldi et al. 1977; Berendsen and Broekkamp 1987; Berendsen et al. 1990; Protais et al. 1995; Millan et al. 1997), the **cholinergic** (Yamada and Furukawa 1980; Fujikawa et al. 1996b), the **GABAergic** (Zarrindast et al. 1995), the **NO system** (Melis et al. 1995, 1996, 1997a,b), and **steroid** as well as **peptide hormones** (Bertolini and Baldari 1975; Bertolini et al. 1978; Holmgren et al. 1980; Berendsen and Nickolson 1981; Berendsen and Gower 1986; Gully et al. 1995) are involved (Argiolas and Melis 1998).

PROCEDURE

Naive male Wistar rats, weighing 220–280 g, are housed under controlled 12 h light-dark cycle with free access to standard food pellets and tap water. Rats are pretreated with subcutaneous injection of the antagonist 30 min prior to injections of the agonist, such as apomorphine (0.02 to 0.25 mg/kg s.c.) or physostigmine (0.02 to 0.3 mg/kg s.c. or i.p.). After administration of the agonist, rats are placed in individual transparent Perspex cages. A mirror is placed behind the row of observation cages to facilitate observation of the animals for penile erections and yawns. Yawning is a fixed innate motor pattern characterized by a slow, wide opening of the mouth. A penile erection is considered to occur when the following behaviours are present: repeated pelvic thrusts immediately followed by an upright position, an emerging, engorged penis which the rats proceeds to lick while eating the ejaculate. The number of penile erections and yawns is counted for 30 min following the last injection.

EVALUATION

The results are expressed as the mean number of yawns and of penile erections per group ±SEM The statistical significance is determined by comparing the results of each group with the results of the relevant control group using a non-parametric rank sum test.

CRITICAL ASSESSMENT OF THE METHOD

Ferrari et al. (1993) published some evidence that yawning and penile erection in rats underlie different neurochemical mechanisms. Nevertheless, the procedure can be regarded as an useful behavioral tool to study putative antipsychotic activity of new compounds.

MODIFICATIONS OF THE METHOD

Two sublines of Sprague Dawley rats were bred for high- and low yawning frequency in males (Eguibar and Moyaho 1997).

Apomorphine produced more yawning in Sprague Dawley rats than in F344 rats (Tang and Himes 1995)

Sato-Suzuki et al. (1998) evoked yawning by electrical or chemical stimulation in the paraventricular nucleus of anesthetized rats.

The yawning – penile erection syndrome in rats can be elicited by injections of 50 ng NMDA or AMPA (Melis et al. 1994, 1997b) into the paraventricular nucleus of the hypothalamus or intracerebroventricular injection of 50 ng oxytocin (Melis et al. 1997a) or ACTH (Genedani et al. 1994; Poggioli et al. 1998) or α-MSH (Vergoni et al. 1998).

Champion et al. (1997), Bivalacqua et al. (1998) studied the effect of intracavernosal injections of adrenomedullin and other peptide hormones on penile erections in **cats**.

Dopaminergic influences on male sexual behavior of **rhesus monkeys** were studied by Pomerantz (1990, 1992).

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E.5.3.6 Inhibition of mouse jumping

PURPOSE AND RATIONALE

Lal et al. (1975) described a jumping response in mice after administration of L-dopa in amphetamine pretreated animals where the number of jumps can be objectively counted. The mouse jumping is due to dopaminergic overstimulation similar to that seen in rats when stereotypy is induced by higher doses of amphetamine. The phenomenon can be blocked by neuroleptics.

PROCEDURE

Male CD-1 mice weighing 22–25 g are injected with 4 mg/kg d-amphetamine sulfate, followed 15 min later by an i.p. injection of 400 mg/kg L-dopa. The mice spontaneously begin to jump at a high rate. A median of 175 jumps can be observed in these mice during 60 min. Since mice do not show any jumping after saline administration, the responses after drug administration are specific and can be measured automatically through a pressure-sensitive switch closure or properly positioned photoelectric beam disruptions. Test compounds are administered 60 min prior to L-dopa injection.

EVALUATION

Jumps of mice treated with test drugs or standard are counted and expressed as percentage of jumps in amphetamine/L-dopa treated animals. Using various doses, ED_{50} values with 95% confidence limits are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method has been found to be sensitive and rather specific for neuroleptic drugs.

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E.5.3.7 Antagonism against MK-801 induced locomotion and falling in mice

PURPOSE AND RATIONALE

MK-801, a non-competitive NMDA antagonist, induces a characteristic stereotypy in mice marked by locomotion and falling behavior through both dopamine dependent and dopamine independent mechanisms (Carlson and Carlson 1989; Verma and Kulkarni 1992). Antipsychotic agents dose-dependent antagonize this MK-801 induced behavior.

PROCEDURE

Male CD-1 mice (20–30 g) are individually placed in activity boxes lined with wire mesh flooring and allowed to acclimate for 60 min. The animals are then dosed with compounds 30 min prior to subcutaneous administration of MK-801 at 0.2 mg/kg. The mice are observed for locomotion and the presence of falling behavior 15 min following MK-801 administration.

EVALUATION

ED_{50} values and 95% confidence limits are calculated by the Litchfield and Wilcoxon method.

MODIFICATIONS OF THE METHOD

Deutsch and Hitri (1993), Rosse et al. (1995) described methods to measure the MK 801-induced explosive behavior in mice, called “popping”.

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E.5.3.8 Inhibition of apomorphine-induced emesis in the dog

PURPOSE AND RATIONALE

The blockade of centrally acting dopaminergic mechanisms is considered to play a major role in suppression of psychotic reactions in schizophrenia. Apomorphine, regarded as a direct dopaminergic agonist, produces a pronounced emetic effect in dogs and the blockade of apomorphine emesis is used as an indication of dopaminergic blockade. However, although both antiemetic activity and antipsychotic activity are thought to be due to dopaminergic blockade, the sites of action are in different brain areas and there is a lack of complete correlation of these activities.

PROCEDURE

Adult beagle dogs of either sex are used in treatment groups of three to nine dogs/dose. The dogs are given the test compounds in a gelatin capsule; they are then dosed with 0.15 mg/kg apomorphine s.c. at various intervals after administration of the test compound. The dogs are first observed for overt behavioral effects, e.g., pupillary response to light, changes in salivation, sedation, tremors, etc; then, after the administration of apomorphine, the dogs are observed for stereotypic sniffing, gnawing and the emetic response. Emesis is defined as wretching movements followed by an opening of the mouth and either attempted or successful ejection of stomach content.

EVALUATION

If the experimental compound is anti-emetic in the primary screen, the dose is progressively lowered to obtain a minimal effective dose or an ED_{50} value. The ED_{50} values for haloperidol and chlorpromazine were found to be 0.06 mg/kg p.o. and 2.0 mg/kg p.o., respectively. Clozapine was not effective at doses between 2 and 10 mg/kg. p.o.

CRITICAL ASSESSMENT OF THE METHOD

The method has been extensively used by several laboratories. However, since non-classical neuroleptics like clozapine did not show pronounced activity the test has been abandoned. Moreover, tests in higher animals like dogs are limited due to regional regulations.

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E.5.3.9 Purposeless chewing in rats

PURPOSE AND RATIONALE

Purposeless chewing can be induced in rats by directly acting cholinergic drugs or cholinesterase inhibitors (Rupniak et al. 1983), which can be blocked by antimuscarinic agents. The chewing behavior has been proposed to be mediated through central M_2 receptors rather than via central M_1 sites (Stewart et al. 1989). Chewing can also be induced by chronic administration of neuroleptics in rats (Clow et al. 1979; Iversen et al. 1980). Purposeless chewing is mediated by dopaminergic and nicotinic mechanisms.

PROCEDURE

Male albino rats are housed 10 per cage at room temperature and kept on a 12 h light-dark cycle. For the experiments, rats are placed individually in a large glass cylinder (height 30 cm, diameter 20 cm) at 21 ±1 °C and allowed to habituate for 15 min before injection of drugs. The antagonists, e.g. sulpiride or mecamylamine as standards, are given at different doses 30 min before treatment either with 0.01 mg/kg nicotine or 1 mg/kg pilocarpine i.p. Number of chewings are counted by direct observation immediately after drug administration. The results are presented as number of chews in a 30 min period.

EVALUATION

Analysis of variance (ANOVA), followed by Newman-Keuls tests, are used to evaluate the significance of the results obtained. $P < 0.05$ is considered as significant.

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E.5.3.10 Single unit recording of A9 and A10 midbrain dopaminergic neurons

PURPOSE AND RATIONALE

Interactions with central nervous system dopamine pathways are crucial for the expression of antipsychotic effects seen with clinically effective neuroleptics. These interactions also have a role in the expression of several of the neurological side effects seen with these agents. Extracellular single unit recording techniques of rat A9 (substantia nigra) and A10 (ventral tegmental area) dopamine neurons show that after acute treatment with neuroleptics the number of spontaneously firing cells is increased in both areas. After repeated treatment (21 days) a decrease was found with all neuroleptics in the A10 neurons, whereas in the A9 cell only compounds with clinically evident extrapyramidal side effects induced a decrease. Clozapine which is believed not to produce extrapyramidal side effects resulted in the depolarization inactivation of A10 neurons but not A9 cells. The method provides a prediction of a compound's anti-psychotic potential as well as potential neurological side effects (Chiodo and Bunney 1983).

PROCEDURE

Male Wistar rats weighing 280–360 g are anesthetized with chloralhydrate intraperitoneally. The animal is mounted in a stereotaxic apparatus (Kopf, model 900). The cranium is exposed, cleaned of connective tissue and dried. The skull overlying both the substantia nigra (A9: anterior (A) 3 000–3 400 µm, lateral (L) 1 800–2 400 µm from lambda), and the ventral tegmental area (A10: A 3 000–3 400 µm, L 400–1 000 µm from lambda) (Paxinos and Watson 1986) is removed. Using the dura as point of reference, a micropipette driven by a hydraulic microdrive is lowered through the opening of the skull at vertical 6 000–8 500 µm. Spontaneously firing dopamine neurons within both the substantia nigra and the ventral tegmental area are counted by lowering the electrode into twelve separate tracks (each track separated from the other by 200 µm) in each region. The sequence of these tracks is kept constant, forming a block of tissue which can be reproducibly located from animal to animal.

Extracellular neuronal signals are sampled using a single barrel micropipette approximately one µm at its tip, and filled with 2 M NaCl saturated with 1% pontamine sky blue dye (*in vitro* impedance between 5 and 10 MΩ). Electrical potentials are passed through a high-impedance preamplifier and the signal is sent to a window discriminator which converts potentials above background noise levels to discrete pulses of fixed amplitude and duration. Only cell whose electrophysiological characteristics match those previously established for midbrain dopamine neurons are counted. In an anesthetized rat, a neuron is considered to be dopaminergic if it displays a triphasic positive-negative-positive spike profile of 0.4 to 1.5 mV amplitude and 2.5 ms duration, firing in an irregular pattern of 3 to 9 Hz with occasional bursts characterized by progressively decreasing spike amplitude and increasing spike duration.

At the end of each experiment, the location of the last recorded track tip is marked by passing 25 microampere cathodal current through the recording micropipet barrel for 15 min in order to deposit a spot of dye. The rat is sacrificed, the brain is then removed, dissected and frozen on a bed of dry ice. Frozen serial sections (20 µm in width) are cut, mounted and stained with cresyl violet and examined using a light microscope.

Animals pretreated with vehicle prior to neuronal sampling serve as controls. For animals that are used in an acute single-unit dopamine neuron sampling assay, test compounds are administered intraperitoneally one hour prior to the beginning of dopamine neuron sampling. For animals used in a chronic single-unit dopamine sampling assay, the compounds are administered once a day for 21 days, and dopamine neuron sampling is begun 2 h after the last dose on the 21st day.

EVALUATION

Drug treatment groups are compared to vehicle groups with a one-way ANOVA with a post hoc Neuman-Keuls analysis for significance.

MODIFICATIONS OF THE METHOD

Nybäck et al. (1975) tested the influence of tricyclic antidepressants on the spontaneous activity of norepinephrine-containing cells of the locus caeruleus in anesthetized rats.

Scuvée-Moreau and Dreese (1979) studied the effect of various antidepressant drugs on the firing rate of locus caeruleus and dorsal raphe neurons of the anesthetized rat with extracellular microelectrodes.

Using the method of single-unit recording of spontaneous firing of locus caeruleus neurons in rats, Cedarbaum and Aghajanian (1977) studied the inhibition by micro-iontophoretic application of catecholaminergic agonists.

Marwaha and Aghajanian (1982) examined in single unit studies the actions of adrenoceptor antagonists at *alpha*-1 adrenoceptors of the dorsal raphe nucleus and the dorsal lateral geniculate nucleus and *alpha*-2 adrenoceptors of the nucleus locus caeruleus.

Mooney et al. (1990) studied the organization and actions of the noradrenergic input to the superior colliculus of the hamster using micro-iontophoretic techniques together with extracellular single unit recording.

Bernardini et al. (1991) studied *in vitro* with brain slices of mice the amphetamine-induced and spontaneous release of dopamine from A9 and A10 cell dendrites.

Santucci et al. (1997) investigated the effects of synthetic neurotensin receptor antagonists on spontaneously active A9 and A10 neurones in rats.

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E.5.3.11 *In vivo* voltammetry

PURPOSE AND RATIONALE

Various groups (Lane et al. 1979, 1987, 1988; Blaha and Lane 1983, 1984, 1987; Crespi et al. 1984; Marsden et al. 1984; Maidment and Marsden 1987a,b; Armstrong-James and Millar 1979, 1984; Kawagoe et al. 1993) described *in vivo* voltammetry as an electrochemical technique that uses carbon fiber microelectrodes stereotactically implanted in brain areas to monitor monoamine metabolism and release. De Simoni et al. (1990) reported on a miniaturized opto-electronic system for telemetry of *in vivo* voltammetric signals in freely moving animals.

PROCEDURE

Carbon fibre working electrodes are made from pyrolytic carbon fibres supported in a pulled glass capillary (Armstrong-James and Millard 1979; Sharp et al. 1984) and electrically pretreated for simultaneous recording of ascorbic acid DOPAC and 5-HIAA (Crespi et al. 1984).

Male Sprague Dawley rats weighing 270–340 g are anesthetized with a 2–3% halothane O_2/NO_2 mixture (1 : 1) and held in a stereotactic frame. Reference and auxiliary electrodes are positioned on the surface of

the dura through 1 mm holes drilled in the cranium and held in place with dental cement. Holes, approx. 2 mm in diameter, are drilled in the cranium above the left or right nucleus accumbens and contralateral anterior striatum, and the underlying dura is broken with a hypodermic needle. A working electrode is lowered in one of the above regions and cemented in place. A second electrode is then implanted in the remaining structure. The coordinates, measured from the bregma, are as follows: nucleus accumbens–rostro-caudal +3.4 mm; medio-lateral ±1.4 mm; dorso-ventral –7 mm; striatum–rostro-caudal +2.8 mm; medio-lateral ±2.6 mm; dorso-ventral –5.5 mm.

Drugs are injected subcutaneously. Voltammograms are recorded using a Princeton Applied Research 174A polarographic analyzer alternatively from each region every 5 min and after a 1 h stabilization period.

EVALUATION

Voltammetric data are expressed as percentage changes from pre-injection control values using the mean of the last 6 peak heights before administration of drug as the 100% value. However, statistical analysis of the data is carried out on the absolute peak heights using a paired Student's *t*-test to compare 6 pre-injection control peak heights with those after administration of drug at selected time points.

MODIFICATIONS OF THE METHOD

Swiergiel et al. (1997) constructed voltammetric probes from stainless steel and fused silica tubing sheathing carbon fibers and compared them with commercially available glass-sealed IVEV-5 electrodes. This type of electrodes can be easily manufactured and does not require any special equipment.

Parada et al. (1994, 1995) described a triple-channel swivel suitable for intracranial fluid delivery and microdialysis experiments which can be equipped with three electrical channels for in-vivo voltammetry and measurement of intracranial temperature with a thermocouple.

Frazer and Daws (1998) used electrodes coated with a perfluorinated ion exchange resin (Nafion®) to assess serotonin transporter function *in vivo* by **chronoamperometry** whereby voltage is applied to the electrode in a pulsed manner and the current obtained measured as a function of time.

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E.6
Antidepressant activity

E.6.0.1
General considerations

The first antidepressant drugs were detected by serendipity in clinical trials. Iproniazid was developed for the treatment of tuberculosis. The observation of mood-elevating effects was followed by the detection of the inhibition of the enzyme monoamine oxidase. During clinical investigation of phenothiazine analogs as neuroleptics, imipramine was found to be relatively ineffective in agitated psychotic patients but showed remarkable benefit in depressed patients. Later on, inhibition of uptake of biogenic amines was found to be the main mechanism of action resulting in downregulation of β-receptors (Vetulani et al. 1976). Influence on α_2-adrenoreceptors (Johnson et al. 1980) was discussed as well. Several lines of preclinical and clinical evidence indicate that an enhancement of 5-HT-mediated neurotransmission might underlie the therapeutic effect of most antidepressant treatments (Blier and de Montigny 1994).

Animal models of depression have been reviewed by Porsolt et al. (1991), Panksepp et al. (1991), Willner and Muscat (1991).

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E.6.1 *In vitro* methods

E.6.1.1 Inhibition of [^{3}H]-norepinephrine uptake in rat brain synaptosomes

PURPOSE AND RATIONALE

As shown by Hertting and Axelrod (1961) the neuronal re-uptake mechanism for norepinephrine is the most important physiological process for removing and inactivating norepinephrine in the synaptic cleft. This uptake is inhibited by cocaine, certain phenylethylamines and antidepressants. This mechanism is considered as one of the most important modes of action of antidepressants leading to receptor down-regulation. In the brain, the hypothalamus shows the highest level and greatest uptake of noradrenaline. Therefore, this region is used for testing potential antidepressant drugs.

PROCEDURE

Tissue preparation

Male Wistar rats are decapitated and the brains rapidly removed. The hypothalamic region is prepared, weighed, and homogenized in 9 volumes of ice-cold 0.32 M sucrose solution using a Potter-Elvejhem homogenizer. The homogenate is centrifuged at 1 000 *g* at 0–4 °C for 10 min. The supernatant is decanted and used for the uptake experiments.

Assay

200 µl of tissue suspension are incubated with 800 µl 62.5 nM ^{3}H-norepinephrine in Krebs-Henseleit bicarbonate buffer and 20 µl of the appropriate drug concentration (or the vehicle) at 37 °C under a 95% O_2/5% CO_2 atmosphere for 5 min. For each assay, 3 tubes are incubated with 20 µl of vehicle at 0 °C in an ice bath. After incubation all tubes are immediately centrifuged at 4 000 *g* for 10 min. The supernatant fluid is aspirated and the pellets dissolved adding 1 ml of solubilizer (Triton X-100 + 50% ethanol, 1 : 4). The tubes are vigorously shaken, decanted into scintillation vials, and counted in 10 ml of liquid scintillation cocktail. Active uptake is the difference between cpm at 37 °C and 0 °C.

EVALUATION

The percent inhibition at each drug concentration is the mean of 3 determinations. IC_{50} values are derived from log-probit analysis. IC_{50} values for the standard drugs desipramine and nortriptyline are around 20 nM.

MODIFICATIONS OF THE METHOD

Pacholczyk et al. (1991) described the expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter.

Tejani-Butt (1992) recommended [3H]nisoxetine as radioligand for quantitation of norepinephrine uptake sites.

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E.6.1.2 Inhibition of [^{3}H]-dopamine uptake in rat striatal synaptosomes

PURPOSE AND RATIONALE

High affinity, saturable, temperature and sodium-dependent transport of ^{3}H-dopamine has been observed in various tissue preparations from different brain regions. The area striata has a high content of dopamine and is suitable for uptake experiments. The ^{3}H-dopamine uptake is inhibited by cocaine, certain phenylethylamines and antidepressants like nomifensine and bupropion, but not by tricyclic antidepressants. The test can be used to characterize the mode of action of antidepressant drugs.

PROCEDURE

Tissue preparation

Male Wistar rats are decapitated and the brains rapidly removed. Corpora striata are prepared, weighed and

homogenized in 9 volumes of ice cold 0.32 M sucrose solution using a Potter-Elvejhem homogenizer. The homogenate is centrifuged at 1 000 *g* at 0–4 °C for 10 min. The supernatant is decanted and used for the experiments.

Assay

100 µl of tissue suspension are mixed with 900 µl 55.5 nM ^{3}H-dopamine solution in Krebs-Henseleit bicarbonate buffer and 20 µl of drug solution in appropriate concentration (or the vehicle as control). The tubes are incubated at 37 °C under a 95% O_2/5% CO_2 atmosphere for 5 min. For each assay, 3 tubes are incubated with 20 µl of vehicle at 0 °C in an ice bath. After incubation all tubes are immediately centrifuged at 4 000 *g* for 10 min. The supernatant fluid is aspirated and the pellets dissolved by adding 1 ml of solubilizer (Triton X-100 + 50% ethanol, 1 : 4). The tubes are vigorously shaken, decanted into scintillation vials, and counted in 10 ml liquid scintillation counting cocktail. Active uptake is the difference between cpm at 37 °C and 0 °C.

EVALUATION

The percent inhibition at each drug concentration is the mean of 3 determinations. IC_{50} values are derived from log-probit analyses. IC_{50} values for nomifensine are 460 nM, but >20 000 nM for tricyclic antidepressants.

MODIFICATIONS OF THE METHOD

Elsworth et al. (1993) differentiated between cocaine-sensitive and -insensitive dopamine uptake in various brain areas.

Cloning and pharmacological characterization of rat, bovine, and human dopamine transporters have been described (Giros et al. 1991; Kilty et al. 1991; Shimada et al. 1991; Usdin et al. 1991; Giros et al. 1992).

Binding characteristics of the dopamine transporter were studied (Reith et al. 1992; Rothman et al. 1992).

[^{3}H]-3β-(*p*-fluorophenyl)tropan-2β-carboxylic acid methyl ester ([^{3}H]WIN 35,428) is used as ligand for the dopamine transporter (Carroll et al. 1992; Cline et al. 1992).

[^{3}H]GBR12 935 (1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine) was used as labelled ligand by Richfield (1991), Nakachi et al. (1995).

Cocaine receptors are specifically labeled with [^{3}H]WIN 35,428 indicating the role of the dopamine transport system in mediating the behavioral effects and the abuse of cocaine (Madras et al. (1989).

Laruelle et al. (1993) reported **single photon emission computed tomography (SPECT)** imaging of dopamine and serotonin transporters in nonhuman primates.

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E.6.1.3 Inhibition of [^{3}H]-serotonin uptake in synaptosomes

PURPOSE AND RATIONALE

Some authors have suggested that patients with serotoninergic hypofunction constitute a subgroup of depression and claim that altered serotoninergic function determines the mood changes associated with affective disorders. A number of clinically effective antidepressants block the reuptake of 5-HT. ^{3}H-5-HT transport in brain has been found to be saturable, sodium- and temperature-dependent, to be inhibited by several agents, such as ouabain, tryptamine analogs, and tricyclic antidepressants. Apparently, the 5-HT uptake can be differentiated from catecholamine uptake. Therefore, the test can be used to detect compounds that inhibit serotonin uptake into rat brain synaptosomes and may be potential antidepressants.

PROCEDURE

Tissue preparation

Male Wistar rats are decapitated and the brains rapidly removed. Either the whole brain minus cerebellum or the hypothalamus is weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose solution using a Potter-Elvejhem homogenizer. The homogenate is centrifuged at 1 000 *g* at 0–4 °C for 10 min. The supernatant is decanted and used for further uptake experiments.

Assay

Two hundred µl of tissue suspension are mixed with 800 µl 62.5 nM ^{3}H-5-HT solution in Krebs-Henseleit bicarbonate buffer and 20 µl of drug solution in the appropriate concentration (or the vehicle as control). The tubes are incubated at 37 °C under 95% O_2/5% CO_2 atmosphere for 5 min. For each assay, 3 tubes are incubated with 20 µl of the vehicle at 0 °C in an ice bath. After incubation all tubes are immediately centrifuged at 4 000 *g* for 10 min. The supernatant is aspirated and the pellets are dissolved by adding 1 ml of solubilizer (Triton X100 + 50% ethanol, 1 + 4). The tubes are vigorously shaken, decanted into scintillation vials, and counted in 10 ml of liquid scintillation counting cocktail. Active uptake is the difference between cpm at 37 °C and 0 °C.

EVALUATION

The percent inhibition of each drug concentration is the mean of 3 determinations. IC_{50} values are calculated from log-probit analyses. Standard drugs, such as chlorimipramine show IC_{50} values in the order of 10 nM for ^{3}H-5-HT uptake in rat synaptosomes from hypothalamus.

CRITICAL ASSESSMENT OF THE METHOD

Whereas many antidepressants inhibit the uptake of serotonin *in vitro*, some drugs like tianeptine were reported to enhance serotonin uptake in *ex vivo* experiments. Therefore, it seems doubtful to relate the therapeutic effect seen in man to a single mechanism of action observed *in vitro*.

MODIFICATIONS OF THE METHOD

Hallstrom et al. (1976) studied the platelet uptake of 5-hydroxytryptamine and dopamine in patients with depression.

Cloning of functional serotonin transporters has been described by Blakely et al. (1991) and by Hoffman et al. (1991).

MODIFICATIONS OF THE METHOD

The role of serotonin in the mode of action of antidepressant drugs has been discussed by Hyttel and Larsen (1985), Åsberg and Mårtensson (1993), Hyttel (1994), Blier and de Montigny (1997), Keane and Soubrié (1997).

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E.6.1.4 Binding to monoamine transporters

PURPOSE AND RATIONALE

There is substantial clinical and experimental evidence that lesions in the serotonergic and noradrenergic systems are responsible for depression and that antidepressant treatment can reverse these alterations (Leonard 2000). Monoamine transporters are principle targets of widely used therapeutic drugs including antidepressants, psychostimulants and the addictive drug cocaine (Madras et al. 1996; Fleckenstein et al. 1999). The termination of neurotransmission is achieved by rapid uptake of the released neurotransmitter by high-affinity neurotransmitter transporters. Most of these transporters are encoded by a family of genes (Na^+/Cl^- transport-ers) having a similar membrane topography of 12 transmembrane helices. An evolutionary tree revealed five distinct subfamilies: GABA-transporters, monoamine transporters, amino acid transporters, "orphan" transporters and bacterial transporters (Nelson 1998).

Tatsumi et al. (1997) described the pharmacological profile of antidepressants at human monoamine transporters.

PROCEDURE

Expression of human transporters

The human serotonin transporter cDNA is directionally ligated into the expression vector pRc/CMV and transfected into HEK293 (human embryonic kidney) cells

by the Ca^{2+} method. The human dopamine transporter cDNA is directionally ligated into the expression vector pcDNA3 and transfected into HEK293 cells, also by the Ca^{2+} method.

Cell culture

The cell lines are grown, passaged, and harvested in 150 mm Petri dishes with 17.5 ml of Dulbecco's modified Eagle's medium containing 0.1 mM nonessential amino acid solution, 5% fetal clonebovine serum product and 1 U/µl penicillin and streptomycin solution. They are incubated in 10% CO_2, 90% air at 37 °C and 100% humidity. The selecting antibiotic geneticin sulfate (250 µg/ml) is used continuously for culture of cells expressing the norepinephrine transporter.

Membrane preparations

For the preparation of the homogenates, the medium is removed by aspiration. The cells are washed with 4 l modified Puck's D1 solution (solution 1) (Pfenning and Richelson 1990) and then incubated for 5 min at 37 °C in 10 ml solution 1 and 100 mM EGTA. Afterwards cells are removed from the surface by scraping with a rubber spatula, placed in a centrifuge tube, and collected by centrifugation at 110 *g* for 5 min at 4 °C. The supernatants are decanted. The pellets are resuspended in the respective binding assay buffer by use of a Polytron (Brinkman Instruments, Westbury, NY) for 10 s at setting 6. The mixture is then centrifuged at 35 600 *g* for 10 min at 4 °C. The pellets are suspended in the same volume of the respective buffer and the centrifugation is repeated. The supernatants are decanted and the final pellets are suspended in the respective buffer and stored at −80 °C until assayed. The final protein concentration is determined by the Lowry assay using bovine serum albumin as standard.

Radioligand binding assays

[^{3}H]imipramine
binding to human serotonin transporter

Radioligand binding assays are performed by a modification of the method of O'Riordan et al. (1990) with a binding buffer containing 50 mM Tris, 120 mM NaCl, and 5 mM KCl (pH 7.4). Compounds to be tested are dissolved in 5 mM HCl and run in duplicate over at least 11 different concentrations against 1 nM [^{3}H]imipramine (specific activity 46.5 Ci/mmol) with 15 µg/tube membrane protein for 30 min at 22 °C. Nonspecific binding is determined in the presence of 1 µM imipramine. With the use of a 48-well Brandel cell harvester, the assay is terminated by rapid filtration through a GF/B filter presoaked with 0.2% polyethylenimine. The filter strips are rinsed five times with ice-cold 0.9% NaCl. Finally, each filter is placed in a scintillation vial containing 6.5 ml of Redi-Safe (Beckman Instruments, Ful-lerton, CA) and counted in a liquid scintillation counter.

[^{3}H]nisoxetine
binding to human norepinephrine transporter

Radioligand binding assays are performed by a modification of the method of Jayanthi et al. (1993) in binding buffer containing 50 mM Tris, 300 mM NaCl and 5 mM KCl (pH 7.4). [^{3}H]nisoxetine (specific activity 85.0 Ci/mmol, from Amersham, Arlington Hts., IL) at 0.5 nM is incubated with competing drugs and 25 µg/tube membrane protein for 60 min at 22 °C. Nonspecific binding is determined in the presence of 1 µM nisoxetine. The remainder of the assay is exactly as described above.

[^{3}H]WIN35 428
binding to human dopamine transporter

Radioligand binding assays are performed using a modification of the method of Pristupa et al. (1994) in a binding buffer containing 50 mM Tris and 120 mM NaCl (pH 7.4). [^{3}H]WIN35 428 (Dupont New England Nuclear, Boston MA, specific activity 83.5 Ci/mmol) at 1 nM is incubated with competing drugs and 30 µg/tube membrane protein for 120 min at 4 °C. Nonspecific binding is determined in the presence of 10 µM WIN35 428. The remainder of the assay is exactly as described above.

EVALUATION

The data are analyzed using the LIGAND program (Munson and Rodbard 1980) for calculation of K_D values and Hill coefficients.

MODIFICATIONS OF THE METHOD

Using the same methods, Tatsumi et al. (1999) described the pharmacological profile of several neuroleptics at human monoamine transporters.

Gu et al. (1994) constructed stable cell lines expressing transporters for dopamine, norepinephrine and serotonin using parental LLC-PK_1 cells which do not express any of these neurotransmitter transporters.

Meltzer et al. (1997) described inhibitors of monoamine transporters using dopamine- and serotonin-transporter assays. Membranes were prepared from coronal slices from caudate-putamen of brain from adult cynomolgus monkeys.

Owens et al. (1997) measured the affinity of several antidepressants and their metabolites for the rat and human serotonin and norepinephrine transporters.

Inazu et al. (1999) characterized dopamine transport in cultured rat astrocytes.

Siebert et al. (2000) used rat neuronal cultures and transfected COS-7 cells to characterize the interaction

of haloperidol metabolites with neurotransmitter transporters.

Sato et al. (2000) studied the selective inhibition of monoamine neurotransmitter transporters by synthetic local anesthetics using cloned transporter cDNAs with transient functional expression in COS cells and stable expression in HeLa cells.

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E.6.1.5 Antagonism of p-chloramphetamine toxicity by inhibitors of serotonin uptake

PURPOSE AND RATIONALE

p-Chloramphetamine causes selective toxicity to serotonin neurons. At 10 mg/kg i.p. p-chloramphetamine (p-CA) causes long-term decreases in 5-HT, 5HIAA, [^{3}H]-5-HT-uptake and tryptophan hydroxylase. After acute administration of p-CA, serotonin uptake into synaptosomes isolated from whole brain is reduced for several hours and returns to control values between 1 and 2 days, after which there is a marked long-lasting decrease due to toxic destruction of 5-HT neurones. The initial behavioral effects of p-CA are due to release of dopamine and serotonin. The serotonergic toxicity of p-CA requires active transport into 5-HT neurones where a cytotoxic intermediate compound is formed. Therefore, compounds which block 5-HT uptake will prevent this toxicity. Antagonism of the long-term p-CA induced reduction of synaptosomal ^{3}H-5-HT *in vitro* uptake is a highly useful index of a compound's ability to inhibit 5-HT re-uptake *in vivo*.

PROCEDURE

Reagents

Two mg/ml dextrose and 0.30 mg/ml iproniazid phosphate are added to Krebs-Henseleit bicarbonate buffer. The mixture is aerated for 1 h with carbogen.

Serotonin creatinine sulfate (Sigma Chemical Co) as a 0.1 mM stock solution in 0.01 N HCl is used to dilute the specific activity of the radiolabeled 5-HT.

A solution of 62.5 nM ^{3}H-5-HT (5-[1,2-^{3}H(N)]-hydroxytryptamine creatinine sulfate, specific activity 20–30 Ci/mmol, New England Nuclear) is prepared in Krebs-Henseleit bicarbonate buffer.

(d,l)-p-Chloramphetamine (Regis Chemical Co) is dissolved in 0.9% NaCl at a concentration of 10 mg/ml.

Drug treatment

Groups of 8 male Wistar rats, weighing 150–200 g are injected intraperitoneally with saline or molar equivalent doses of the test drug. For initial studies, the dose given is 37,5 µmol/kg (equivalent to 10 mg/kg desipramine base). After 30 min, 4 rats from each group are injected with saline or with p-CA 10 mg/kg i.p. Three days after treatment, the rats are sacrificed. For multiple dosing, the rats are pretreated with the test drug twice up to 4 times in 2 h intervals.

Tissue preparation

The rats are decapitated and the brains rapidly removed. Whole brain minus cerebellum is weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose using a Potter-Elvejhem homogenizer. The homogenate is centrifuged at 1 000 *g* at 0–4 °C for 10 min. The crude synaptosomal supernatant is decanted and used for uptake experiments.

Assay

800 µl Krebs-Henseleit bicarbonate buffer + [^{3}H]-5-HT
200 µl Tissue suspension

Tubes are incubated at 37 °C under a 95% O_2/5% CO_2 atmosphere for 5 min. For each assay, 3 tubes are incubated at 0 °C in an ice bath. After incubation all tubes are immediately centrifuged at 4 000 *g* at 0–4 °C for 10 min. The supernatant fluid is aspirated and the pellets dissolved by adding 1 ml of solubilizer (Triton X-100 and 50% ethanol, 1 : 4, v/v). The tubes are vigorously vortexed, decanted into scintillation vials, and counted in 10 ml of aqueous (Liquiscint) scintillation counting cocktail. Active uptake is the difference between cpm at 37 °C and 0 °C.

EVALUATION

The percent protection is calculated according to the following formula:

$$\%\ \text{protection} = \frac{cpm[(sal\ /\ sal) - (sal\ /\ pCA)]}{cpm[(sal\ /\ sal) - (sal\ /\ pCA)]} - \frac{cpm[(sal\ /\ sal) - (drug\ /\ pCA)]}{cpm[(sal\ /\ sal) - (sal\ /\ pCA)]}$$

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E.6.1.6 Receptor subsensitivity after treatment with antidepressants: Simultaneous determination of the effect of chronic anti-depressant treatment on b-adrenergic and 5-HT$_2$ receptor densities in rat cerebral cortex

PURPOSE AND RATIONALE

The catecholamine and indolamine systems are thought to be involved in affective disorders such as depression. The effect of antidepressants on biogenic amine re-uptake *in vitro* is immediate; whereas the onset of clinical activity is delayed and parallels more closely the time course of receptor changes measured in animal studies. Therefore, the experiment is designed to determine the *in vivo* effects of chronic (10 days) treatment with known and potential antidepressants on the β-receptor, as a measurement of noradrenergic interaction, and on the 5-HT$_2$ receptor as a measurement of serotoninergic interaction in the rat brain. Both receptor densities are measured in cortical tissue from the same animal and compared after treatment with test compounds at doses similar to those of standard drugs.

PROCEDURE

Drug treatment

Groups of 4 male Wistar rats, receiving food and water at libitum are maintained on a 12-h diurnal light cycle and given i.p. injections twice daily for 10 days with saline or molar equivalent doses of the experimental drugs (equivalent to 10 mg/kg imipramine). Twenty-four hours after the last dose, the rats are decapitated and the cerebral cortices split along the mid-sagittal sinuses. One half is used for the (^{3}H)-DAH assay and the other half is used for the (^{3}H)-spiroperidol assay. This protocol allows the determination of effects of antidepressants on β-receptors and 5-HT$_2$-receptors in cerebral cortical tissue from the same animal.

Reagents

- (–)-[propyl-1,2,3-^{3}H]Dihydroalprenololol hydrochloride (45–52 Ci/mmol) is obtained from New England Nuclear.
- (±)-propranolol HCl is obtained from Ayerst.
- [Benzene-^{3}H]spiroperidol (20–35 Ci/mmol) is obtained from New England Nuclear.

Tissue preparation

^{3}H-Dihydroalprenolol (^{3}H-DHA) binding

The cerebral cortices are dissected free, weighed and homogenized in 50 vol of ice-cold 0.05 M Tris buffer, pH 8.0. This homogenate is centrifuged at 40 000 *g* and the supernatant decanted. The pellet is resuspended and again centrifuged at 40 000 *g*. The final pellet is resuspended in 0.05 M Tris buffer, pH 8.0. This tissue suspension is then stored on ice until use. The final tissue concentration is 10 mg/ml.

^{3}H-Spiroperidol binding

The cerebral cortices are dissected, weighed and homogenized in 50 vol of 0.05 M Tris buffer, pH 7.7, and then centrifuged at 40 000 *g* for 15 min. The supernatant is discarded, the pellet resuspended and again centrifuged at 40 000 *g*. The final pellet is resuspended in 50 vol of 0.05 M Tris buffer, pH 7.7 and stored in an ice bath. The final concentration in the assay is 10 mg/ml.

Assay

^{3}H-DAH binding

380 µl H_2O
50 µl 0.5 M Tris buffer pH 8.0
20 µl vehicle (for total binding) or 50 µM propranolol (for nonspecific binding)
50 µl ^{3}H-DAH stock solution
500 µl tissue suspension

The tissue homogenates are incubated for 15 min at 25 °C with varying concentrations of ^{3}H-DHA (0.25–4.0 nM). With each ligand concentration triplicate samples are incubated with 1 µM propranolol under identical conditions to determine nonspecific binding. The total added ligand is determined by counting 50 µl of each (^{3}H)-DHA concentration. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed 3 times with 5 ml of ice cold 0.05 M Tris buffer, pH 8.0. The filters are counted in 10 ml liquid scintillation cocktail.

^{3}H-Spiroperidol binding

50 µl 0.5 M Tris-physiological salts
380 µl H_2O
20 µl H_2O (for total binding) or 0.25 mM methysergide (for nonspecific binding)
50 µl ^{3}H-spiroperidol stock solution
500 µl tissue suspension

The tissue homogenates are incubated for 10 min at 37 °C with varying concentrations of ^{3}H-spiroperidol (0.1–3.0 nM). With each ligand concentration, triplicate samples are incubated in the presence of 5 µM methysergide under identical conditions to determine nonspecific binding. The total added ligand is determined by counting 50 µl of each (^{3}H)-spiroperidol concentration. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed 3 times with 5 ml of ice cold 0.05 M Tris buffer, pH 7.7. The filters are transferred to scintillation vials and counted in 10 ml of liquid scintillation cocktail.

EVALUATION

Specific binding is defined as the difference in CPM in the presence or absence of excess "cold" ligand. The free ligand concentration is the difference between the total added and the specifically bound fraction at each concentration. The equilibrium binding constants (K_d and B_{max}) are determined by Scatchard analyses using least square regression analysis of the binding data. The Scatchard plot shows "bound/free" versus "bound". The K_d value is the reciprocal of the slope and B_{max} is the x-intercept. Significant differences of drug treatment are determined by either Dunnett's or Tukey's test after one way analysis of variance.

MODOFICATIONS OF THE METHOD

Buckett et al. (1988) found a rapid down-regulation of β-adrenoceptors in brains of rats after 3 days of oral treatment with sibutramine.

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E.6.1.7 Measurement of β-adrenoreceptor stimulated adenylate cyclase

PURPOSE AND RATIONALE

Noradrenaline stimulates the β-adrenoreceptor linked adenylate cyclaseβ in rat brain. Reduction of this stimulation after treatment with antidepressants is an indicator for receptor down-regulation.

PROCEDURE

Groups of male Sprague-Dawley rats are treated intraperitoneally twice daily for 14 days with saline, 10 mg/kg desimipramine or the test compound. The rats are decapitated and frontal cortices are removed, and placed into ice-cold Krebs physiological buffer (120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 2 mM KH_2PO_4, 2 mM $MgSO_4$, 25 mM $NaHCO_3$, 100 µM $Na_2S_2O_5$, 25 µM EDTA, and 10 mM glucose), pH 7.4. Cortices are cut 350 × 350 µ at right angles, added to 20 ml Krebs physiological buffer in 50 ml conical flasks, and separated by vortex mixing. The slices are incubated at 37 °C for 1 h in a shaking water bath with the buffer replaced by freshly oxygenated medium every 15 min. After this step, 20 µl [^{3}H]adenine is added to each flask. The flasks are capped, and the slices are incubated for an additional 30 min at 37 °C. This medium is then discarded and the slices are washed with 20 ml fresh buffer. Most of the medium is removed and 30 µl aliquots of the packed slices are pipetted into tubes containing 300 µM 3-isobutyl-1-methylxanthine in 270 µl Krebs physiological buffer. After incubation at 37 °C for 15 min, 30 µl of noradrenaline (final concentration 100 µM) or 30 µl buffer are added to each tube and incubated for an additional 10 min. The reaction is stopped by addition of 0.5 ml 1 M HCl. The tubes are placed in ice for 10 min before addition of 0.5 ml NaOH and then centrifuged at 3 000 *g* for 10 min at 4 °C. The supernatants are transferred to tubes containing [^{14}C]-cyclic AMP (approximately 10 000 cpm) to monitor the recovery of [^{3}H]-cyclic AMP. The radio-labelled cyclic nucleotides are separated by two-stage column chromatography on alumina neutral WN-3 and Dowex AG 50W-X4 anion exchange resin.

EVALUATION

The activity of adenylate cyclase is calculated as the conversion of [^{3}H]-adenine to [^{3}H]-cyclic AMP. After treatment with antidepressants, this conversion rate is

not altered without stimulation by noradrenaline, but significantly reduced in slices treated with the maximally stimulating concentration of 100 µM noradrenaline.

CRITICAL ASSESSMENT OF THE METHOD

Determination of β-adrenoreceptor stimulated adenylate cyclase is another parameter for measurement of down-regulation of adrenoreceptors by chronic treatment with antidepressants.

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E.6.1.8 [^{3}H]Yohimbine binding to α_2-adrenoceptors in rat cerebral cortex

PURPOSE AND RATIONALE

This binding assay is used to investigate the interaction of compounds at central α_2 receptors and may indicate possible modes of action for antidepressant, antihypertensive and other classes of compounds.

[^{3}H]Yohimbine is a selective antagonist for α_2-receptors (Starke et al. 1975). The use of an antagonist radioligand avoids the complexity of saturation curves that can be observed with radio-labeled agonist ligands. Furthermore, a [^{3}H]antagonist label for the α_2 receptor permits a better evaluation of α_2/α_1 selectivity than a [^{3}H]agonist label, since α_1 affinities are measured with a [^{3}H]antagonist ([^{3}H]WB 4 101).

Chronic treatment with desipramine has been shown to decrease the binding of [^{3}H]DHA to rat brain cortical β-receptors. Some investigators have reported that co-administration of yohimbine causes β-receptor down-regulation to occur after fewer antidepressant treatments (Johnson et al. 1980; Scott and Crews 1983). Therefore, it may be of interest to investigate compounds with yohimbine-like properties as antidepressant candidates themselves or in conjunction with antidepressant candidates.

PROCEDURE

Reagents

1. 20-fold concentrated buffer:
   2.36 M NaCl137.92 g/liter
   100 mM KCl7.45 g/liter
   200 mM glucose 36.03 g/liter in 0.5 M Tris, pH 7.4
2. 2-fold concentrated buffer:
   - fold dilution of reagent 1 in deionized water
3. Standard buffer:
   - 20-fold dilution of reagent 1 in deionized water
4. [Methyl-^{3}H] yohimbine (72–86 Ci/mmol) is obtained from New England Nuclear
   - For IC_{50} determinations: [^{3}H] Yohimbine is diluted to 40 nM in deionized H_2O
   - Fifty µl of this solution is a 2-ml reaction volume gives a final concentration of 1 nM
5. L-NE bitartrate is made up to 10 mM in 0.01 N HCl. 20 µl of this solution gives a final concentration of 100 µM in 2 ml of reaction mixture.
6. Test compounds. For most assays, a 10 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-4} to 10^{-7} M. Six or seven concentrations are used routinely.

Tissue preparation

Male Wistar rats are decapitated and their brains rapidly removed. The cortices are weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle (0.009–0.010 cm clearance). The homogenate is centrifuged at 1 000 *g* for 10 min (approximately

3 000 rpm using the Sorvall RC-5 centrifuge with heads SS-34 or SM-24). The supernatant is then recentrifuged at 17 000 *g* for 20 min (approximately 12 500 rpm using the Sorvall RC-5 centrifuge, and heads SS-34 or SM-24). The pellet (P_2) is resuspended in the original volume of 0.32 M sucrose and stored on ice.

Binding assay

430 µl H_2O
1 000 µl 2-fold concentration buffer (reagent 2)
20 µl drug or 10 mM L-NE bitartrate or vehicle
50 µl ^{3}H-yohimbine (reagent 4)
500 µl tissue

Tubes are vortexed and incubated at 25 °C for 10 min. Bound [^{3}H]yohimbine is captured via filtration under reduced pressure. The filters are washed three times with 5 ml aliquots of buffer (reagent 3). The filters are then counted in 10 ml of Liquiscint scintillation fluid.

EVALUATION

Specific binding of [^{3}H]yohimbine is the difference between total bound (in the presence of vehicle) and nonspecifically bound (in the presence of 100 µM L-NE bitartrate). Percent inhibition of specific [^{3}H]yohimbine binding is calculated for each concentration of test drug and IC_{50} values determined by computer-derived log-probit analysis.

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E.6.1.9 Test for anticholinergic properties by [^{3}H]-QNB binding to muscarinic cholinergic receptors in rat brain

PURPOSE AND RATIONALE

Several tricyclic antidepressants exert considerable anticholinergic effects which limit the therapeutic use in some patients. Amitryptiline has the greatest incidence of these side effects and is the most potent in binding to muscarinic receptors. Desipramine, which exhibits less incidence of atropine like side effects shows a lower affinity for muscarinic receptors. Since there is no evidence that the anticholinergic effects contribute to the therapeutic efficacy, antidepressant drugs with low anticholinergic effects are desired.

Quinuclidinyl benzilate (QNB) is a specific muscarinic cholinergic antagonist in both peripheral and central tissues. The binding characteristics of ^{3}H-QNB were first described by Yamamura and Snyder (1974), who showed that this ligand was displaced by muscarinic antagonists, but not by nicotinic or non-cholinergic drugs. The levorotatory isomer being more potent than the racemate is used as the radioactive ligand.

PROCEDURE

Reagents

L-[Benzilic-4,4'-^{3}H]-quinuclidinyl benzilate (30–40 Ci/mmol) is obtained from New England Nuclear. For IC_{50} determinations [^{3}H]-QNB is made up to a concentration of 40 nM and 50 µl is added to each tube (yielding a final concentration of 1 nM in the assay).

Atropine sulfate is obtained from Sigma Chemical Co.

Test compounds: For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, so that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M.

Tissue preparation

Male Wistar rats are killed by decapitation and their brains rapidly removed. After removal of the cerebellum, each brain is homogenized in 10 volumes of ice-cold 0.32 M sucrose in a Potter-Elvejhem glass homogenizer. The homogenate is then centrifuged at 1 000 *g* for 10 min and the pellet discarded. The resultant supernatant is further dispersed and used for [^{3}H]-QNB binding studies. Specific binding is 20% of the total added ligand and approximately 95% of the total bound ligand.

Assay

100 µl 0.5 M Na/K phosphate buffer, pH 7.4 (134 g $Na_2HPO_4 \cdot 7\,H_2O + 68$ g KH_2PO_4)
780 µl H_2O
50 µl ^{3}H-QNB stock solution
20 µl vehicle (for total binding) or 2 mM atropine (for nonspecific binding)
1 000 µl 0.05 M Na/K phosphate buffer (1 : 10 diluted) pH 7.4
50 µl tissue suspension

Tissue homogenates are incubated for 60 min at 25 °C with 1 nM ^{3}H-QNB and varying drug concentrations. With each binding assay, triplicate samples containing 2 µM atropine sulfate are incubated under identical conditions to determine nonspecific [^{3}H]-QNB binding. After incubation, the samples are cooled and then rapidly filtered through glass filters (Whatman GF/B)

under reduced pressure. The filters are washed 3 times with 5 ml of ice-cold phosphate buffer and then placed in scintillation vials. After the addition of 10 ml of counting cocktail, radioactivity is assayed by liquid scintillation spectrophotometry.

EVALUATION

Specific [^{3}H]-QNB binding is determined by the difference between total [^{3}H]-QNB and bound radioactivity in the presence of 2 μM atropine sulfate. Data are con-verted to percent specific bound [^{3}H]-QNB displaced by test drugs and IC_{50} values obtained from computer derived log-probit analysis. The inhibition at each drug concentration is the mean of triplicate determinations.

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E.6.1.10 Monoamine oxidase inhibition: Inhibition of type A and type B monoamine oxidase activities in rat brain synaptosomes

PURPOSE AND RATIONALE

The mood-elevating effects of the antituberculosis drug iproniazid have been observed clinically. The mode of action was elucidated to be the inhibition of the enzyme monoamine oxidase. This was followed by wide use of monoamine oxidase inhibitors for the treatment of depression. However, side effects due to interaction with dietary amines have been observed. The biological role of monoamine oxidase is to regulate the levels of endogenous amines (norepinephrine, dopamine and serotonin) and exogenously administered amines. Based on different substrate and inhibitor specificities two forms of monoamine oxidase (A and B) were described. Dopamine and tyramine are substrates for both types, serotonin and epinephrine are substrates for type A, and β-phenylethylamine and benzylamine are substrates for type B. Iproniazid and tranylcypromine are nonselective inhibitors, clorgyline is a selective inhibitor of type A, deprenyl and pargyline are selective inhibitors of type B. It has been suggested that treatment with selective blockers of type B results in less detrimental food interactions.

PROCEDURE

Tissue preparation

Male Wistar rats weighing 150–250 g are sacrificed and the brains rapidly removed. Whole brain minus cerebellum is homogenized in 9 volumes of ice-cold, phosphate-buffered 0.25 M sucrose, using a Potter-Elvejhem homogenizer. The homogenate is centrifuged at 1 000 *g* for 10 min and the supernatant decanted and recentrifuged at 18 000 *g* for 20 min. The resulting pellet (P_2) is resuspended in fresh 0.25 M sucrose and recentrifuged at 18 000 *g* for 20 min. The washed pellet is resuspended in the original volume of 0.25 M sucrose and serves as the tissue source for mitochondrial monoamine oxidase.

Assay

50 μl 0.5 M PO_4 buffer, pH 7.4
450 μl H_2O
100 μl H_2O or appropriate drug concentration
200 μl tissue suspension

The tubes are preincubated for 15 min at 37 °C and the assay is started by adding 100 μl of substrate (^{14}C-5-HT or ^{14}C β-phenylethylamine) at 10 s intervals. The tubes are incubated for 30 min at 37 °C and the reaction is stopped by the addition of 0.3 ml of 2 N HCl. Tissue blank values are determined by adding the acid before the substrate. Seven ml of diethylether are added, the tubes are capped and shaken vigorously for 10 min to extract the deaminated metabolites into the organic phase, which is separated from the aqueous phase by centrifugation at 1 000 *g* for 5 min. A 4 ml aliquot of the ether layer is counted in 10 ml of liquid scintillation counting cocktail.

EVALUATION

The percent inhibition at each drug concentration is the mean of triplicate determinations. IC_{50} values are determined by log-probit analyses.

For example, deprenyl shows IC_{50}-values of 3.9×10^{-6} against MAO A and 3.0×10^{-8} against MAO B.

MODIFICATION OF THE METHOD

Colzi et al. (1992) measured dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) outflow after a reversible MAO-A inhibitor with a brain microdialysis technique in rats.

Frankhauser et al. (1994) tested the interaction of MAO inhibitors and dietary tyramine by measurement of peak systolic blood pressure in conscious rats. The increase of blood pressure after oral application of tyramine was potentiated by pretreatment with MAO inhibitors. Reversible MAO inhibitors could be differentiated from non-selective, irreversible MAO inhibitors.

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E.6.2 Behavioral tests

E.6.2.1 Catalepsy antagonism in chicken

PURPOSE AND RATIONALE

Observations about cataleptic behavior in chicken have first been described more than 300 years ago (Schwenter 1636; Kircher 1646), and again reported about 100 years ago (Czermak 1873; Heubel 1877; Verworn 1898). This phenomenon was used by Vogel and Ther (1963) as a simple method to detect antidepressants besides other central stimulants.

PROCEDURE

Adult white Leghorn chicken are used. The animal is grasped with both hands whereby the left hand pushes the chicken slightly down and the right hand supports the animal from the ventral side. Immediately, the chicken is turned on its back and hold with the right hand for 1 min. Usually, cataleptic numbness occurs immediately. The cataleptic state can be sustained by slight pushing the head of the animal on the table. After 1 min the right hand is carefully withdrawn. The chicken remains in the cataleptic state for several min up to 1 h. The cataleptic rigor is interrupted by noise or fast movements of the observer. Clapping of the hands above the head arouses the chicken which jumps up and runs away. The chicken is always aroused by pulling on the wings. The animals are pretested in order to be sure about the cataleptic behavior of an individual chicken. As already found by previous investigators, the experiment can be repeated several times. Control studies showed that in untreated animals the phenomenon could be elicited 6 times every 30 min during a period 5 days. After the control experiments, the animals are injected i.p. with the test compound or the vehicle. The test is performed 4 times every 30 min during 2 h.

EVALUATION

The test is considered to be positive if the cataleptic rigor does not occur after treatment or is interrupted spontaneously within 1 min at least twice during the 2 h test period. The suppression of the cataleptic phenomenon is the criterion for a positive response. Furthermore, the arousal after hand-clapping or pulling on the wings is recorded in order to register central sedative effects. In order to obtain dose – response curves 12 animals per group are treated with various doses. ED_{50} values are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The specificity of the method has been tested. Antidepressant agents like imipramine and ethyltryptamine-acetate or other monoamino-oxydase-inhibitors and d-desoxyephedrine show a dose dependent effect. Moreover, the effects were dependent on time. ED_{50} values decreased on consecutive days after imipramine and monoamino-oxydase inhibitors but not after d-desoxyephedrine. Therefore, the test can be considered as specific for central stimulants allowing the possibility to distinguish between antidepressants and central stimulants of the amphetamine type.

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E.6.2.2 Despair swim test

PURPOSE AND RATIONALE

Behavioral despair was proposed as a model to test for antidepressant activity by Porsolt et al. (1977, 1978). It was suggested that mice or rats forced to swim in a restricted space from which they cannot escape are induced to a characteristic behavior of immobility. This behavior reflects a state of despair which can reduced by several agents which are therapeutically effective in human depression.

PROCEDURE

Male Sprague-Dawley rats weighing 160–180 g are used. They are brought to the laboratory at least one day before the experiment and are housed separately in Makrolon® cages with free access to food and water. Naive rats are individually forced to swim inside a vertical Plexiglas cylinder (height: 40 cm; diameter: 18 cm, containing 15 cm of water maintained at 25 °C). Rats placed in the cylinders for the first time are initially highly active, vigorously swimming in circles, trying to climb the wall or diving to the bottom. After 2–3 min activity begins to subside and to be interspersed with phases of immobility or floating of increasing length. After 5–6 min immobility reaches a plateau where the rats remain immobile for approximately 80% of the time. After 15 min in the water the rats are removed and allowed to dry in a heated enclosure (32 °C) before being returned to their home cages. They are again placed in the cylinder 24 h later and the total duration of immobility is measured during a 5 min test. Floating behavior during this 5 min period has been found to be reproducible in different groups of rats. An animal is judged to be immobile whenever it remains floating passively in the water in a slightly hunched but upright position, its nose just above the surface. Test drugs or standard are administered one hour prior to testing. Since experiments with the standard drug (imipramine) showed that injections 1, 5 and 24 h prior the test gave the most stable results in reducing floating these times are chosen for the experiment.

EVALUATION

Duration of immobility is measured in controls and animals treated with various doses of a test drug or standard. Antidepressant drugs, but also stimulants like amphetamine and caffeine, reduce duration of immobility. Dose-responses can be evaluated.

MODIFICATIONS OF THE METHOD

Wallach and Hedley (1979) reported positive results with antihistamines and Giardina and Ebert (1989) with an ACE-inhibitor. Differentiation is achieved by the simultaneous evaluation of motor activity.

Cervo and Samanin (1987) suggested potential antidepressant activities of a selective serotonin$_{1A}$ agonist based on anti-immobility activity in the forced swimming test in rats without effect on open-field activity.

Nishimura (1988, 1989, 1993) published a modification of the forced swim test using straw suspension in the water tank. The apparatus used was a vertical glass cylinder (height: 40 cm, diameter: 18 cm) equipped with 4 pieces of straw (length 24 cm, diameter: 0.4 cm) that were suspended from above. The cores of the straws were filled with cotton rope. The straws were painted black from the surface of the water to a height of 10 cm. The apparatus was filled with water to a height of 15 cm and maintained at 25 °C. Thirty min after treatment with drugs or saline, the rats were placed in the apparatus

without straw suspension and the total duration of immobility measured for a period of 5 min. Immediately thereafter, 4 pieces of straw were suspended and the total duration of immobility in the following 5-min observation period again measured.

Buckett et al. (1982) described an automated apparatus for behavioral testing of typical and atypical antidepressants in **mice**. A multichannel system can test 10 mice simultaneously. Each mouse is placed in the beam of a Doppler radar head and horn assembly. The moving mouse causes reflections of a frequency differing from the transmitted signal. Within the Doppler head these reflected waves are mixed with a proportion of transmitted waves to produce a difference signal proportional to the activity of the mouse within the beam. The output of each Doppler head is fed to an amplifier whose gain has been calibrated to compensate for differences in sensitivity between individual heads. The method is claimed to eliminate human error and bias and to allow the testing of large numbers of compounds.

Alpermann et al. (1992) used a slightly modified behavioral despair test in mice. Sixty min after administration of the test compounds, the animals are placed in glass cylinders containing water up to a height of 10 cm (water temperature 22–24 °C). From the second minute onward, immobility of each mouse is rated every 30 s. After 10 observations mean values and standard deviations in each treatment group are calculated. Compared with the immobility score of the control group percent reduction can be calculated.

Nomura et al. (1982) published a modification of the despair swim test in mice involving a small water wheel set in a water tank. Mice placed on this apparatus turned the wheel vigorously but, when they abandoned attempts to escape from the water, the wheel stopped turning. The number of rotations of the water wheel were counted.

Hata et al. (1995) studied the behavioral characteristics of SART (specific alterations of rhythm in temperature)-stressed mice in forced swimming tests and evaluated the effects of anxiolytics and antidepressants.

CRITICAL ASSESSMENT OF THE METHOD

The method has been used and modified by many authors, e.g. Kauppila et al. (1991, van der Heyden et al. 1991).

Advantages of the method are the relative simplicity and the fact that no interaction with other drugs is necessary. Like in other behavioral tests, e.g. the catalepsy test in chicken, not only antidepressants and monoamino-oxydase inhibitors but also central stimulants give positive results.

In a critical review of the forced swimming test, Borsini and Meli (1988) discussed the various modifications and proposed rats to be more suitable than mice for detecting antidepressant activity.

The model is purely behavioral without presuppositions concerning the mechanism of action of potential antidepressants. It is sensitive to a large number of atypical antidepressants otherwise inactive in the more classical tests. The rat version seems to be more selective (fewer false positives) and the mouse version more sensitive (fewer false negatives) (Porsolt et al. 1991).

Natoh et al. (1992) reviewed the theoretical background of the forced swimming test and the various factors which can possibly affect the results.

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E.6.2.3 Tail suspension test in mice

PURPOSE AND RATIONALE

The "tail suspension test" has been described by Steru et al. (1985) as a facile means of evaluating potential antidepressants. The immobility displayed by rodents when subjected to an unavoidable and inescapable stress has been hypothesized to reflect behavioral despair which in turn may reflect depressive disorders in humans. Clinically effective antidepressants reduce the immobility that mice display after active and unsuccessful attempts to escape when suspended by the tail.

PROCEDURE

Male Balb/cJ mice weighing 20–25 g are used preferentially. They are housed in plastic cages for at least 10 days prior to testing in a 12 h light cycle with food and water freely available. Animals are transported from the housing room to the testing area in their own cages and allowed to adapt to the new environment for 1 h before testing. Groups of 10 animals are treated with the test compounds or the vehicle by intraperitoneal injection 30 min prior to testing. For the test the mice are suspended on the edge of a shelf 58 cm above a table top by adhesive tape placed approximately 1 cm from the tip of the tail. The duration of immobility is recorded for a period of 5 min. Mice are considered immobile when they hang passively and completely motionless for at least 1 min.

EVALUATION

The percentage of animals showing the passive behavior is counted and compared with vehicle treated controls. Using various doses, ED_{50} values can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The tail suspension test has been found to be an easy method to test potential antidepressant compounds. However, it has been reported that several mouse strains are essentially resistant to tail-suspension induced immobility.

MODIFICATIONS OF THE METHOD

Chermat et al. (1986) adapted the tail suspension test to the rat.

Porsolt et al. (1987), Stéru et al. (1987) recommended the use of the automated tail suspension test for the primary screening of psychotropic agents. A specially developed computerized device automatically measures the duration of immobility of 6 mice at one time and at the same time provides a measure of the energy expended by each animal, the power of the movements.

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E.6.2.4 Learned helplessness in rats

PURPOSE AND RATIONALE

Animals exposed to inescapable and unavoidable electric shocks in one situation later fail to escape shock in a different situation when escape is possible (Overmier and Seligman 1967; Maier and Seligman 1976). This phenomenon was evaluated as a potential animal model of depression (Sherman et al. 1979; Martin et al. (1986), Christensen and Geoffroy 1991; Tejedor del Real et al. 1991).

PROCEDURE

Learned helplessness is produced in male Sprague-Dawley rats (300 g) by exposure to electric shock (0.7 mA) for 1 h on a schedule of 10 s of shock/min. The apparatus is a $30 \times 45 \times 30$ cm box with a grid floor. At a height of 20 cm above the floor, a platform (7.5×7.5 cm) can be inserted through one side wall to allow a jump-up escape response. The platform is not available during training. After the appropriate treatment, the animals are tested for acquisition of a jump-up escape in the same apparatus. At the beginning of a trial, the platform is pushed into the box and a 0.4 mA shock initiated. Shock is terminated in 10 s if the animal has not escaped onto the platform by this time. If an escape response occurred, the animal is allowed to remain on the platform for the duration of 10 s, then returned to the grid floor. Ten such trials with an intertrial interval of 20 s are given. In a naive control group of rats, this training resulted in 80% acquiring learned helplessness behavior. Drugs are given before the training and the test period.

EVALUATION

A drug is considered to be effective, if the learned helplessness is reduced and the number of failures to escape is decreased. Imipramine was found to be active only after repeated applications. A benzodiazepine was effective, whereas chlorpromazine was ineffective.

CRITICAL ASSESSMENT OF THE METHOD

The "learned helplessness" in the rat can be regarded as an additional measure for antidepressant activity in addition to other tests. The test is time consuming and its specificity is questionable.

MODIFICATIONS OF THE METHOD

Vaccheri et al. (1984) used an apparatus with an lever to be pressed to interrupt the shock. Giral et al. (1988), Porsolt et al. (1990) Simiand et al. (1992) used shuttle-boxes for escape.

Curzon et al. (1992) described a similar rat model of depression. Rats were tested in an open field after being restrained by taping them to wire grids for two h on the preceding day. The reduced activity is antagonized by antidepressant drugs.

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E.6.2.5 Muricide behavior in rats

PURPOSE AND RATIONALE

Horovitz et al. (1965) described a selective inhibition of mouse-killing behavior in rats (Karli 1956; Karli et al. 1969) by antidepressants. The test can be used to evaluate antidepressants such as tricyclics and MAO-inhibitors.

PROCEDURE

Male Sprague-Dawley rats (300–350 g) are isolated for 6 weeks in individual cages. They have access to food and water ad libitum. One mouse is placed into the rat's cage. About 10 to 30% of rats kill the mouse by biting the animal through the cervical cord. Only rats consistently killing mice within 5 min after presentation are used for the test. The mice are removed 15 to 45 s after they have been killed in order to prevent

the rats from eating them. Drugs are injected i.p. to the rats before the test. Mice are presented 30, 60 and 120 min after drug administration.

EVALUATION

Failure to kill a mouse within 5 min is considered inhibition of muricidal behavior. Performing dose-response experiments, the ED_{50} is defined as the dose which inhibits mouse killing in 50% of the rats.

MODIFICATIONS OF THE METHOD

Injections of 5,7-dihydroxytryptamine into the lateral hypothalamus increased mouse-killing behavior in rats (Vergnes and Kempf 1982).

Molina et al. (1985) considered rats isolated for at least one month which do not kill mice in a 30 min test period as spontaneous non-killer rats. In these animals, mouse-killing behavior could be induced by i.p. injection of 150 mg/kg p-chlorophenylalanine daily for 2 days or by electrolytic lesions in the dorsal and median raphe.

McMillen et al. (1988) studied the effects of housing and muricidal behavior on serotonergic receptors and interactions with novel anxiolytic drugs.

CRITICAL ASSESSMENT OF THE METHOD

The mouse-killing behavior is inhibited not only by antidepressants but also by central stimulants like d-amphetamine (Horovitz et al. 1966; Sofia 1969a,b), some antihistamines (Barnett et al. 1969), and some cholinergic drugs (McCarthy 1966; Vogel and Leaf 1972; Wnek and Leaf 1972). Neuroleptics and benzodiazepines are active only in doses which impair motor performance.

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E.6.2.6 Behavioral changes after neonatal clomipramine treatment

PURPOSE AND RATIONALE

Vogel et al. (1988, 1990a), Hartley et al. (1990) reported that neonatally administered clomipramine produces changes in adult rats that resembles endogenous depression in man, based on earlier observations by Mirmiran et al. (1981), Rodriguez-Echandia and Broitman (1983). This phenomenon has been studied by several research groups in many respects (Neill et al. 1990; Vogel et al. 1990b–e, 1996a,b; Velazquez-Moctezuma and Diaz-Ruiz 1992; Yavari et al. 1993; Maudhuit et al. 1995, 1996; Prathiba et al. 1995, 1997, 1998, 1999; Feenstra et al. 1996; Kinney et al. 1997; Bonilla-Jaime et al. 1998; Dwyer and Rosenwasser 1998).

PROCEDURE

Three days after birth, all male pups of Sprague Dawley mother rats are cross-fostered and all female pups are killed. Cross-fostering consists of removing all pups from their biological mothers and placing them with another lactating female (the non-biological foster mother). Each litter is divided into two groups of approximately equal number. Each half of the litter is placed with a different lactating female (the foster mother), and each foster mother receives half the pups

from two litters. All pups with each foster mother receive the same treatment (clomipramine or saline) and the two pup groups of each original litter are assigned to different treatments. Thus, each original pup litter contributes equally to experimental and control groups. Each pup is injected subcutaneously between the shoulder blades with 15 mg/kg clomipramine hydrochloride or saline vehicle twice daily on postnatal days 8 through 21. At one month of age the pups are weaned and housed as litters until approximately 3 weeks prior testing, at which time they are individually housed.

All testing is conducted in a Coulbourn operant conditioning chamber (Lehigh Valley, PA) placed in a quiet area. The floor rods are connected with a shock generator and a shock scrambler.

Behavioral testing is commenced at 3 months postnatally. Each test pair consists of one clomipramine-treated and one vehicle-treated rat. Prior to testing, all clomipramine- and saline-treated rats are paired by body weight (within 5 grams) to diminish size differences that could affect behavioral results. Pairs remain the same throughout the testing.

For evaluation of drugs, clomipramine-treated rats are administered subcutaneously for 4 days twice daily saline, or standard drug (10 mg/kg imipramine), or test drug.

Tests are done daily for 4 days. On the first day, the animals are placed in the chamber for 12 min habituation. On days 2–4, the sessions start with a 2 min habituation period followed by 10 min of shock delivery. Shocks (1.33 mA, 0.5 s duration) are delivered on a variable schedule with a minimum of 5 and a maximum of 10 s shock intervals. This results in a total of 70–80 shocks within the 10-min session.

Two observers score simultaneously. Each watches one rat of the pair. The rats are identified by a red mark on the fur. The behaviors produced are almost totally in response to shock delivery; both animals are almost immobile between the shocks. Both observers are blind to the treatment conditions of the animals.

Offensive and defensive behaviors are scored including the following 4 behaviors:

1. An upright position which is part of a mutual upright posture in which the dominant rat towers over the submissive rat. The submissive rat rears on its hindlegs, with the head positioned at an upward angle, the forepaws extended toward the attacking animal, and the ventral surface of the body continually facing the opponent.
2. In the offensive crouch, the dominant animal turns its flank towards the subordinate; the submissive crouch is characterized by freezing in a motionless crouching position.
3. Mounting behavior is frequently seen and is the same as seen in the male prior to copulating with a female.
4. Leaps in response to the shock which are directed to the other rat are scored as aggressive responses.

Three defensive or submissive behaviors were observed:

1. Defensive upright posture;
2. submissive crouch;
3. a supine position in submission to the dominant rat.

In this system each offensive behavior by one rat is partly defined by a defensive behavior of the other rat and vice versa.

EVALUATION

The individual behaviors of the treatment and control groups are listed for offensive and defensive behavior on days 1, 2, and 3 and the total of scores calculated for each group. Analysis of variance is applied to offensive, defensive, difference (offensive minus defensive) and total (offensive plus defensive behavior scores. Rats treated postnatally with clomipramine have significantly fewer offensive and significantly more defensive behaviors. This effect is ameliorated by treatment with antidepressant drugs.

CRITICAL ASSESSMENT OF THE TEST

Not only clomipramine, but also other psychotropic drugs induce changes in behavior of adult rats after treatment in neonatal age (Drago et al. 1985; Frank and Heller 1997; Hansen et al. 1998), however, clomipramine induces the most pronounced effects (Velazquez-Moctezuma et al. 1993; Vogel and Hagler 1996; Hansen et al. 1997). Specificity of the procedure to evaluate potential antidepressant compounds remains to be established.

MODIFICATIONS OF THE METHOD

Neonatal treatment with clomipramine not only reduces shock induced aggression, but also induces REM sleep abnormalities (Mirmiran et al. 1981; Vogel et al. 1990b,c; Frank and Heller 1997), decreases intracranial self-stimulation (Vogel et al. 1990d), increases activity in open-field tests in adulthood (Hartley et al. (1990, Prathiba et al. 1997), increases immobility in the forced swim test (Velazquez-Moctezuma and Diaz-Ruiz 1992), reduces adult male rat sexual behavior (Neill et al. 1990; Velazquez-Moctezuma et al. 1993; Vogel et al. 1996; Bonilla-Jaime et al. 1998,), influences the hypothalamic-pituitary-adrenal axis in adult rats (Feenstra et al. 1996; Prathiba et al. 1998, 1999).

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E.6.2.7 Antidepressant-like activity in differential-reinforcement of low rate 72-second schedule

PURPOSE AND RATIONALE

The differential-reinforcement-of low-rate (DRL) 72-s schedule has been recommended for evaluation of antidepressant drugs (O'Donnell and Seiden 1983).

PROCEDURE

Male Sprague Dawley rats weighing 350–450 g are housed in suspended wire cages in rooms maintained at 21–23 °C and 30% to 40% relative humidity having free access to laboratory chow except during experi-

mental sessions. Water is provided after each daily session in order to maintain body weights.

Apparatus

Model C Gerbrand operant-conditioning chambers (O'Donnell and Seiden 1983) or Coulbourn operant chambers (Pollard and Howard 1986) are used. A lever that operates a microswitch is mounted on one wall 3 cm from the side, 2.5 cm above a grid floor and 6.5 cm from an access port for a dipper that provides 0.025 ml of water. A houselight is mounted on the opposite wall. A downwards force equivalent to approximately 15 g operates the lever, constituting a response. When the schedule requirements are met, the dipper is lifted from a water trough to an opening in the floor of the access port for 4 s, constituting a reinforcer.

Rats are water-deprived for 22 h before each session. Each rat is initially trained under an alternative fixed-ratio 1, fixed-time 1-min schedule for water reinforcement. Thus, each response is reinforced, and reinforcement is also provided every min if no responding occurs. All rats are then placed under a DRL (differential-reinforcement-of-low-rate) 18 s schedule for 3 weeks. The schedule requirement is then increased to 72 s (DRL 72-sec). When performances on the DRL 72-sec schedule are stabilized (approximately 6 weeks), drug treatments are initiated.

Each group of 6 to 10 rats is subjected to a dose-response determination for one ore more drugs. Drugs are administered one h before testing by intraperitoneal injection.

EVALUATION

Data are expressed as percentages of non-injected controls. The control values are the response and reinforcement rates on those days immediately preceding the test day. The number of responses and reinforcers per session at each drug dose are tested for statistically significant differences from control values with paired t-tests using a two tailed criterion of statistical significance.

CRITICAL ASSESSMENT OF THE TEST

The method is rather time consuming. Moreover, the specificity as screening method for antidepressants has been challenged (Pollard and Howard 1986).

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E.6.3
Tests for antidepressant activity based on the mechanism of action

E.6.3.1
Potentiation of norepinephrine toxicity

PURPOSE AND RATIONALE

Antidepressants block the re-uptake of biogenic amines into nervous tissue. In this way, the toxic effects of norepinephrine are potentiated.

PROCEDURE

Male NMRI mice (22–25 g) are randomly assigned to test groups of 10 subjects. The test drug, the standard or the vehicle are given orally 1 h prior to the s.c. injection of the sublethal dose of 3 mg/kg noradrenaline. The groups of 10 mice are placed into plastic cages with free access to food and water.

EVALUATION

The mortality rate is assessed 48 h post-dosing. ED_{50}, or dose which causes death of 50% of the treated subjects, is calculated by means of linear regression analysis.

CRITICAL ASSESSMENT OF THE METHOD

Several antidepressants block not only the uptake of noradrenaline, but also of dopamine and of serotonin. By definition, this test can only measure the noradrenaline-uptake inhibition.

One of the earliest observations on the pharmacology of antidepressants was by Sigg (1959), who showed that imipramine, as distinguished from other tricyclic substances known at this time, produced a profound potentiation of the cardiovascular effects of exogenously administered catecholamines in animals.

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E.6.3.2 Compulsive gnawing in mice

PURPOSE AND RATIONALE
In man and in other species, like dogs, apomorphine induces emesis. Treatment of rodents with apomorphine causes compulsive gnawing instead of vomiting. The compulsive gnawing in mice induced by apomorphine is due to dopaminergic stimulation. Centrally acting anticholinergics shift the balance between acetylcholine and dopamine resulting in an enhancement of the apomorphine effect. Therefore, many compounds with psychotropic activity are known to have an apomorphine-synergistic effect. This enhancement is also found after administration of tricyclic antidepressants.

PROCEDURE
NMRI mice with a body weight between 18 and 20 g are injected s.c. with 10 mg/kg apomorphine. At the same time they are treated i.p. or s.c. with the test drug or the vehicle. For testing oral activity the animals are treated 30 min prior to apomorphine injection. Immediately after apomorphine injection 6 mice are placed into a cage $45 \times 45 \times 20$ cm with a wired lid. The bottom of the cage is covered with corrugated paper, the corrugation facing upwards. The mice start to bite into the paper causing fine holes or tear the paper. This behavior is enhanced by antidepressants. The mice remain 1 h in the cage.

EVALUATION
The number of bites into the corrugated paper is evaluated by placing a template upon the paper. The template has 10 rectangle windows divided into 10 areas of the same size. In a total of 100 areas the number of bites is checked. In this way percentage of damaged paper in calculated. Ten mg/kg apomorphine does not increase the biting rate of 5–10% which also occurs in normal animals. Likewise, atropine in doses of 40 and 80 mg/kg s.c. alone does not increase the biting behavior. In contrast, the combination of apomorphine and atropine greatly enhances the occurrence of gnawing. The same is true for antidepressants. For example, a dose of 25 mg/kg imipramine increases the damaged area by 40–70%. Percent gnawing of the test compound is compared with that of the standard.

CRITICAL ASSESSMENT OF THE TEST
Not only antidepressants, but also centrally acting anticholinergics and antihistaminics are active in this test. However, the test has the advantage of simplicity without any pretraining of the animals.

MODIFICATIONS OF THE METHOD
De Feo et al. (1983) reported on possible dopaminergic involvement in biting compulsion of mice induced by large doses of clonidine.

Stereotyped gnawing in mice is induced by methylphenidate (Pedersen and Christensen 1972). The phenomenon is potentiated by various drugs, such as benzodiazepines (Nielsen et al. 1991).

Stereotyped behavior in **guinea pigs** induced by apomorphine or amphetamine consisting in continuous gnawing and sniffing of the cage floor was described by Klawans and Rubovits (1972) and used as an experimental model of tardive dyskinesia.

Molander and Randrup (1974) investigated the mechanisms by which L-DOPA induces gnawing in mice.

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E.6.3.3 Apomorphine- induced hypothermia in mice

PURPOSE AND RATIONALE
Apomorphine induces hypothermia in mice which can be prevented by antidepressants.

PROCEDURE
Male NMRI mice (20–22 g) are used and randomly assigned to test groups of 6 subjects. One hour after

oral administration of the test compounds or the vehicle a dose of 16 mg/kg apomorphine is injected s.c. The rectal temperature of each mouse is measured by an electronic thermometer immediately prior to apomorphine administration and 10, 20 and 30 min later. During the entire experiment, subjects are housed in groups of three in glass jars at room temperature.

EVALUATION

A time curve is constructed by plotting the temperature (mean of each group) against time in min. The AUC is calculated for all groups and converted into percent inhibition of apomorphine-induced hypothermia in the control group. An ED_{50} can be calculated by linear regression analysis.

CRITICAL ASSESSMENT OF THE METHOD

Antagonism against apomorphine-induced hypothermia can be regarded as a hint for antidepressant activity. Compounds with a marked noradrenergic or dopaminergic component are active against apomorphine-induced hypothermia but not antidepressants acting mainly through serotoninergic mechanisms.

MODIFICATIONS OF THE METHOD

Cox and Lee (1981) induced hypothermia in rats by intrahypothalamic injection of 5-hydroxytryptamine and recommended this as a model for the quantitative study of 5-hydroxytryptamine receptors.

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E.6.3.4 Tetrabenazine antagonism in mice

PURPOSE AND RATIONALE

Tetrabenazine (TBZ) induces a depletion of biogenic amines (e.g. noradrenaline, dopamine, serotonin) without affecting their de novo synthesis. TBZ depletes noradrenaline from nerve terminals and prolongs re-uptake into the granula. Noradrenaline is degraded by monoamino-oxydase. Antidepressants inhibit the re-uptake of noradrenaline into the nerve terminals and increase thereby the noradrenaline concentration at the receptor site. In this way, the effect of TBZ is antagonized. Therefore, both MAO-inhibitors and tricyclic antidepressants are known to prevent or to antagonize these effects. The prevention of TBZ induced ptosis and catalepsy can be used for evaluation of antidepressants.

PROCEDURE

Groups of 5–10 male NMRI mice (20–22 g) are used. Sixty min after oral or 30 min after i.p administration of the test compound or the vehicle 40 mg/kg i.p. TBZ are injected. The animals are placed into individual cages. The test is started 30 min after TBZ administration and repeated every 30 min up to 2 h. Catalepsy and ptosis are used as criteria. A stair is formed with 2 cork stoppers having 2 steps of 3 cm height. The animals are placed head downwards with their hindlegs upon the top cork. As long as TBZ exerts its cataleptic effect the animals remain in this catatonic state. If the cataleptic effect is not antagonized after a limit of 60 s the animals are placed into a normal position.

EVALUATION

Thirty s after replacement the degree of ptosis is scored: eyes closed = 4, eyes ¾ closed = 3, eyes ½ closed = 2, eyes ¼ closed = 1, eyes open = 0. (Rubin et al. 1957) Similarly, the cataleptic effect is scored according to the duration of catalepsy. Catalepsy more than 60 s = 5, between 30 and 60 s = 4, between 10 and 30 s = 3, between 5 and 10 s = 1, less than 5 s = 0. The scores of the TBZ controls are taken as 100% and the percentage is calculated for the treated animals. Imipramine was found to be effective at a dose of 10 mg/kg s.c. or 20 mg/kg orally.

CRITICAL ASSESSMENT OF THE METHOD

The TBZ antagonism has been found to be a simple and reliable test for evaluation of classical antidepressants.

MODIFICATIONS OF THE METHOD

Ptosis in mice can be induced in a similar way by treatment with reserpine. Mice receive a single oral dose of the test compound followed by subcutaneous administration of 5 mg/kg reserpine 30 min later. Ptosis is observed for the next 1 or 2 h and scored in a similar way as in the TBZ test.

TBZ ptosis can also be elicited in rats. The test procedure and the evaluation is the same as in mice. Tetrabenazine-induced hyperthermia in mice has been proposed as test for antidepressant activity by Gylys et al. (1963).

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E.6.3.5 Reserpine induced hypothermia

PURPOSE AND RATIONALE

Depletion of biogenic amines (noradrenaline, 5-hydroxytryptamine, dopamine) in the brain induces not only catalepsy and ptosis but also hypothermia in rodents. The decrease of body temperature induced by reserpine is antagonized by antidepressants, MAO-inhibitors and central stimulants. The subcutaneous administration of 2 mg/kg reserpine leads to a decrease of core temperature in mice to 20–23 °C after 18 h. The fall in temperature can be antagonized by antidepressants, but also by amphetamine-like drugs. However, the time course is different: tricyclic antidepressants have a slow onset of action and a long lasting effect, whereas amphetamine-like drugs have a quick onset of action and a short-lasting effect.

PROCEDURE

Groups of 5 male NMRI mice (19–21 g body weight) are used. On the day before testing, they are dosed with 2 mg/kg reserpine s.c. They are housed in a climate-controlled animal colony and have free access to food and water. Eighteen hours after reserpine administration, the animals are placed into individual cages. The initial rectal temperature is determined by insertion of an electronic thermometer (e.g. Ellab T-3) to a constant depth of 2 cm. Following administration of the test compound (either i.p. or p.o.), the rectal temperature is measured again at 60 min intervals for 7 h.

EVALUATION

Rectal temperature is recorded every hour. The difference in temperature from vehicle controls is calculated for each time and the maximal difference is scored. The differences are then statistically compared using the *t*-test.

MODIFICATIONS OF THE METHOD

The time course and the administration route can be changed. Male mice are treated with the test drug or the standard 1 h prior to intravenous injection of 2 mg/kg reserpine. Rectal temperature is measured by a rectal thermometer prior and every 60 min up to 6 h The animals are kept in groups of 3 in glass jars at a controlled temperature of 20–21 °C. Using a computer program the area under the curve is calculated by plotting the temperature (mean of each group) before and the decrease after reserpine against time in hours. Areas of treated groups are converted to percentage of controls.

Colpaert et al. (1975), Niemegeers (1975) described the antagonism of antidepressants and other drugs against Ro-4-1 284, a benzoquinolizine derivative which by itself exhibits reserpine-like activities.

CRITICAL ASSESSMENT OF THE METHOD

The test has been proven as a simple and reliable method to detect antidepressant activity. However, the reversal of hypothermia is not specific for antidepressants. The fall in body temperature can also be antagonized by amphetamines, and some antipsychotic agents (chlorpromazine). The different time course of antidepressants (slow onset of action, long-lasting effect) and amphetamine-like drugs (quick onset of action, short-lasting effect) allows differentiation between the two groups of drugs.

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E.6.3.6 5-Hydroxytryptophan potentiation in mice

PURPOSE AND RATIONALE

According to the monoamine hypothesis of depression compounds exert antidepressant activity because they are capable of enhancing central noradrenergic and/or serotoninergic functions. Several antidepressant agents potentiate serotonin effects by a block of the re-uptake of serotonin. DL-5-Hydroxytryptophan is used as the precursor of serotonin. Enzymatic breakdown is inhibited by the MAO-inhibitor pargyline. In mice the characteristic symptom of head-twitches is observed.

PROCEDURE

Groups of 6 male mice (18–30 g) are used. They are treated i.p with the test drug or the vehicle. Thirty min later, the mice receive 75 mg/kg pargyline HCl s.c. Ninety min after pargyline the animals are injected wit 10 mg/kg DL-5-hydroxytryptophan (5-HTP) i.v.

EVALUATION

Animals positively influenced show a characteristic behavior of head twitches. A animal is considered to be positive if it shows head twitches 15 min after 5-HTP injection. Enhancement is observed after treatment with serotonin uptake blockers relative to animals pretreated with pargyline only.

CRITICAL ASSESSMENT OF THE METHOD

The test can be considered as additional evidence for antidepressant activity based on uptake inhibition.

MODIFICATION OF THE METHOD

The head-twitch in mice can also be elicited without a MAO-inhibitor by using higher doses (200 mg/kg) of DL-5-hydroxytryptophan.

Meert et al. (1988) studied partial and complete blockade of 5-hydroxytryptophan (5-HTP)-induced head twitches in the *rat* by serotonin S_2 antagonists.

Niemegeers et al. (1983), Awouters et al. (1988) used *mescaline* induced head twitches in the rat as an *in vivo* method to evaluate serotonin S_2-antagonists. Head twitches were counted for 15 min after intravenous injection of 20 mg/kg mescaline. Twitch counts of less than 2 were considered as inhibition and of less than 2 as blockade of the mescaline response.

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E.6.3.7 5-Hydroxytryptophan potentiation in rats

PURPOSE AND RATIONALE

The inhibition of serotonin re-uptake by some antidepressants can be tested *in vivo* in rats by administration of the precursor 5-hydroxytryptophan and inhibition of its breakdown by the MAO-inhibitor pargyline. In contrast to mice exhibiting head twitches, rats show other symptoms such as continuous forelimb clonus.

PROCEDURE

Groups of 6 male Wistar rats weighing 150–200 g are used. Four hours prior to testing pargyline HCl is injected sc. at a dose of 75 mg/kg. Thirty min before i.p. injection of 1 mg/kg L-5-hydroxytryptophan, test compounds or standards are administered intraperitoneally. Fifteen min after the 5-hydroxytryptophan injection, the animals are observed for 15 min. An animal is considered to be positive if is exhibits continuous forelimb clonus.

EVALUATION

Enhancement is expressed as normalized percent increase relative to the vehicle control. Using various doses, ED_{50} values with 95% confidence limits can be determined by probit analysis.

MODIFICATIONS OF THE METHOD

Hallberg et al. (1985) developed a registration device based on accelerometry in order to accomplish an objective quantification of tremors in conscious unrestrained rats. Tremor intensity was continuously recorded by a small piecoresistive accelerometer mounted on the back of the freely moving rat. The accelerometer was connected to a Grass Polygraph. The integrated signals were further analyzed by a desk-top computer.

The behavior in rats induced by 10 mg/kg L-5-hydroxytryptophan i.p. can be antagonized by compounds having 5-HT_2 antagonist properties (Colpaert and Janssen 1983).

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E.6.3.8 Yohimbine toxicity enhancement

PURPOSE AND RATIONALE

Yohimbine occupies central α_2-receptors, and prevents noradrenaline from binding to these receptors. Compounds with antidepressant properties are known to inhibit physiological inactivation of noradrenaline and other biogenic amines by blocking the re-uptake at nerve terminals. Administration of a test compound with antidepressant properties leads to an increase in noradrenaline concentration. Following the simultaneous administration of yohimbine and antidepressants the animals die of noradrenaline poisoning.

PROCEDURE

Groups of 10 male NMRI mice (25–28 g) are used. Mice are placed in plastic cages and receive the test compound or the vehicle by oral or i.p. administration. Thirty min later, a dose of 25 mg/kg yohimbine (a sublethal dose) is given s.c.

EVALUATION

Mortality rate is assessed 1 h, 2 h, 3 h, 4 h, 5 h, and 24 h after dosing. Lethality in the control group (Yohimbine only) is less than 10%, whereas 10 mg/kg desipramine-HCl causes death in about 90%. Using various doses ED_{50}-values can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The test has been proven as a simple method to detect antidepressants with monoamine uptake inhibiting properties.

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E.6.3.9 Tryptamine seizure potentiation in rats

PURPOSE AND RATIONALE

Monoamino-oxydase (MAO) inhibitors like iproniazid enhance seizures in rats caused by an intravenous infusion of tryptamine HCl. This procedure can be used to elucidate the *in vivo* MAO inhibiting properties of compounds.

PROCEDURE

Groups of 5 male Wistar rats weighing 150–200 g are used. Test compounds, standard or vehicle controls are administered intraperitoneally 0.5; 1; 2; and 4 h prior testing. At the time of testing 5 mg/kg tryptamine HCl freshly dissolved in saline are injected intravenously. Immediately after tryptamine HCl administration, the animals are observed individually for three min for the appearance of clonic "pedalling" movements of the forepaws which is considered a positive response. Frequently, these clonic seizures are preceded by a kyphotic curvature of the spine but this sign does not constitute a positive response.

In addition to the vehicle control group, a series of five positive control animals receiving tranylcypromine at 5 mg/kg i.p. with a 0.5 h pretreatment time are subjected to the test in order to check the effectiveness of the tryptamine HCl solution which is relatively unstable. A 100% response is expected. Fresh tryptamine HCl solution should be prepared hourly as needed.

EVALUATION

The normalized percent potentiation is calculated as follows:

$$100 \times \left[\frac{\%\ \text{animals potent. in drug group}}{1 - \%\ \text{animals potent. in vehicle group}} - \frac{\%\ \text{animals potent. in vehicle}}{1 - \%\ \text{animals potent. in vehicle group}} \right]$$

A dose-response is obtained in the same manner at the peak time of drug effect except that a group size of 10 animals is used and four different doses are tested in addition to the vehicle and the tranylcypromine groups.

An ED_{50} is calculated using probit analysis.

MODIFICATIONS OF THE METHOD

Graham-Smith (1971) described the inhibitory effect of chlorpromazine on the syndrome of hyperactivity produced by L-tryptophan or 5-methoxy-N,N-dimethyltryptamine in rats treated with a monoamine oxidase inhibitor.

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E.6.3.10 Serotonin syndrome in rats

PURPOSE AND RATIONALE

Compounds which stimulate serotonin receptors cause a series of behavioral changes in rats which is called the serotonin syndrome (Jacobs 1976; Green and Heal 1985; Tricklebank 1985) such as head weaving, increased locomotion, forepaw treading, flat posture and lower lip retraction. With increasing knowledge about the subtypes of serotonin receptors these symptoms were defined to be associated with 5-HT_{1A} receptors and their specific agonists (Smith and Peroutka (1986), Blanchard et al. 1993; Yu and Lewander 1997).

PROCEDURE

Time- and dose-dependence of **forepaw treading**, which is scored at 15, 30, 45, and 60 min according to a 3-point scale (0 = absent; 1 = periodic and weak; 2 = continuous) according to Arnt and Hyttel (1989) and Porsolt et al. (1992),

was measured by Deakin and Green (1978), Green et al. (1983), Goodwin and Green (1985), Goodwin et al. (1986), Berendsen and Broekkamp (1990), Schoeffter et al. (1993), Andersson and Larsson (1994), Forster et al. (1995), Kofman and Levin (1995), Lu and Nagayama (1996), O'Connell and Curzon (1966), Gaggi et al. (1997), Kleven et al. (1997), Yu and Lewander (1997),

of **flat body posture** by Deakin and Green (1978), Goodwin and Green (1985), Goodwin et al. (1986), Blanchard et al. (1993), Schoeffter et al. (1993), Andersson and Larsson (1994), Foreman et al. (1993, 1994, 1995), Forster et al. (1995), Kofman and Levin (1995), O'Connell and Curzon (1966), Gaggi et al. (1997), Kleven et al. (1997), Wolff et al. (1997), Yu and Lewander (1997),

of **hind limb abduction** by Deakin and Green (1978), Green et al. (1983), Goodwin and Green (1985), Goodwin et al. (1986), Kofman and Levin (1995),

of **increased motility** by Tricklebank (1985), Forster et al. (1995), Gaggi et al. (1997), O'Neill and Parameswaran (1997),

of **decreased body temperature** by Martin et al. (1992), Schoeffter et al. (1993), Simiand et al. (1993), Foreman et al. (1993, 1994, 1995), Forster et al. (1995), O'Connell and Curzon (1966), Bagdy and To (1997), Wolff et al. (1997), Yu and Lewander (1997),

of **head twitches** by Deakin and Green (1978), Green et al. (1983), Goodwin and Green (1985), Goodwin et al. (1986), Meert et al. (1988), Berendsen and Broekkamp (1990,) Kofman and Levin (1995), Gaggi et al. (1997), Kleven et al. (1997),

of **lower lip retraction**, which is scored according to Berendsen et al. (1989) after 15, 30, 45 min as follows: 0 = lower incisors not or hardly visible (not different from nontreated animals), 0.5 = partly visible, 1 = completely visible,

by Smith and Peroutka (1986), Berendsen et al. (1994, 1997), Porsolt et al. (1992), Blanchard et al. (1993), Andersson and Larsson (1994), Foreman et al. (1993, 1994, 1995), Moore et al. (1993), De Boer et al. (1995), Berendsen et al. (1966), Bagdy and To (1997), Groenink et al. 1997; Kleven et al. (1997), Wolff et al. (1997).

EVALUATION

Scores of each symptom are registered for each test animal. Average values of treated animals are compared with controls treated with vehicle alone. Dose-response and time response curves are evaluated.

CRITICAL ASSESSMENT OF THE TEST

Among the many symptoms of the serotonin syndrome in rats, forepaw treading, flat body posture and lower lip retraction were used by most authors to characterize agonists and antagonists of 5-HT_{1A} receptors.

MODIFICATIONS OF THE METHOD

Trulson et al. (1976) used the serotonin syndrome to test the supersensitivity after destruction of central serotonergic nerve terminals by intracerebral injection of 5,7-dihydroxytryptamine.

Blanchard et al. (1997) described the symptoms of selective activation of 5-HT_{1A} receptors in **mice**.

Locomotor activity of **guinea pigs** was increased in a dose-dependent manner by 5-HT_{1A} receptor agonists (Evenden 1994).

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E.6.3.11
Hypermotility in olfactory-bulbectomized rats

PURPOSE AND RATIONALE

Bilateral olfactory bulbectomy in the rat is associated with changes in exploratory behavior that are reversed by chronic, but not acute treatment with antidepressant drugs (Cairncross et al. 1978, 1979; Leonard and Tuite 1981; Janscár and Leonard 1981).

PROCEDURE

Male Sprague Dawley rats are anesthetized with intraperitoneal injection of 2.5% tribromo-ethanol solution (Cairncross et al. 1977). Following exposure of the skull, a burr hole is drilled at points 7 mm anterior to the bregma and 2 mm either side of the midline at a point corresponding to the posterior margin of the orbit of the eye. The olfactory bulbs are removed by suction and the burr holes filled with hemostatic sponge. Tetramycin powder is then applied to the wound and the skin closed by surgical clips. Sham-operated animals are treated in the same way but although the dura above the bulbs is cut, the bulbs are left intact. The animals are allowed to recover for 14 days after surgery.

For drug evaluation the animals are treated subcutaneously with the test drug or the standard or the vehicle once daily at 09:00 for 14 days. In each experiment sham-operated controls treated with test drug, standard and vehicle are included.

The behavior of the animals is tested from the 12th day onwards. The rats are placed singly in the center of an open field apparatus. Ambulation (no. of squares crossed), rearing (forepaws raised from the floor), grooming and defecation (no. of fecal boli) scores are recorded for an 3 min period of observation.

EVALUATION

The results are analyzed statistically using Student's *t*-test (2 tail test), the significance being set at $P < 0.05$.

CRITICAL ASSESSMENT OF THE METHOD

The bulbectomized rat model has been shown to be highly selective for both typical and atypical antidepressants, however, the procedure is quite time consuming.

MODIFICATIONS OF THE METHOD

Various authors used this model to demonstrate antidepressant-like activity: such as Briley et al. (1996) for milnacipran, a serotonin and noradrenaline uptake inhibitor, Hancock et al. (1995) for A-80 426, an α_2-adrenoceptor antagonist with serotonin uptake blocking activity, McNamara et al. (1995) for the centrally active serotonin agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), Redmont et al. (1997) for dizocilpine (MK-801), Song and Leonard (1994) for the serotonin reuptake inhibitors fluvoxamine and sertraline, Song et al. (1996a) for centrally administered neuropeptide Y, Song et al. (1996b) for the H_1 receptor antagonist terfenadine.

Kelly and Leonard (1994, 1995) studied the effects of potential antidepressants, such as selective serotonin reuptake inhibitors, in olfactory bulbectomized rats.

Redmont et al. (1995) studied the effect of chronic antidepressant administration on the conditioned taste aversion to 8-OHDPAT in the olfactory bulbectomized rat.

Kelly et al. (1997) gave an update of the olfactory bulbectomized rat as a model of depression. Tricyclic antidepressants (amitryptiline, desimipramine), atypical agents (mianserin), selective serotonin reuptake inhibitors (paroxetine, sertraline, fluvoxamine), reversible inhibitors of monoamine oxidase A (moclobemide), as well as putative antidepressants, such as 5-HT_{1A} agonists (zalospirone, ipsapirone), noncompetitive NMDA antagonists (MK-801) and triazolobenzazepines (alprazolam, adinazolam), have demonstrated antidepressant-like activity in this model.

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E.6.3.12 Sexual behavior in male rats

PURPOSE AND RATIONALE

Sexual behavior in male rats is stimulated by 5-HT_{1A} receptors agonists (Ahlenius et al. 1981; Gorzalka et al.

(1990), Foreman et al. 1993, 1994, 1995; Anderson and Larsson 1994; Tallentire et al. 1996; Ahlenius and Larsson 1997; Fernández-Guasti and Rodriguez-Manzo 1997) and inhibited by serotonin receptor antagonists (Mendelson and Gorzalka 1981) and by 5-HT_{1B} receptors antagonists (Fernández-Guasti et al. 1989). The test procedure was described in detail by Arnone et al. (1995).

PROCEDURE

Adult male and ovarectomized female Sprague Dawley rats are housed in sex-separated rooms at 21 ±1 °C in a reversed light-dark cycle with free access to food and water.

Mating behavior

Male rats of two levels of sexual performance are selected for drug testing: sexually naive and sexually experienced. The latter are given 4 mating pretests, twice a week. Only sexually active males that achieve at least two ejaculations per test, are included in the experiments.

The mating tests are performed between 12:30 and 17:00, during the dark phase of the lighting cycle. Drug is administered orally 1 h before the test to the male rat. The animal is allowed to adapt to the test area (60 cm diameter, 50 cm high) illuminated with a dim red light. Each behavioral test starts with the introduction of a stimulus female brought into sexual receptivity by s.c. treatment with estradiol benzoate (10 µg/rat), followed 48 h later by progesterone (500 µg/rat), 4–5 h before testing. The tests end either 20 min later or after the first post-ejaculatory mount (or 2 h later for sexual satiation).

The following behavioral parameters are recorded:

- **Mount and intromission latencies:** time interval from the first introduction of the female to the first mount or intromission, respectively. Mounts are accompanied by an average of three or four brief shallow thrusts, while the intromission, which succeeds this event, is marked by a single deep thrust indicative of penile insertion.
- **Mount frequency:** total number of pre-ejaculatory mounts with and without intromission.
- **Intromission frequency:** total number of pre-ejaculatory intromissions.
- **Ejaculatory latency:** time interval from the first intromission to ejaculation. A total of 1 200 s is scored for the latencies of rats failing to mount, intromit or ejaculate.

Sexual satiation

The mating pattern of the male rat consists of repeated mounts and intromissions, culminating in an ejaculation. The ejaculation is followed by a period of 4–5 min during which time the male remains refractory to sexual stimulation by the female. The sexual activity is thereafter resumed with a new series of mounts and intromissions followed by ejaculation. If uninterrupted, the rat may achieve five to six ejaculations before sexual satiation sets in. Sexually experienced male rats are allowed to copulated with a receptive female for 2 h. The behaviors are recorded on a videotape. The criterion for satiation is that the male fails to mount within 20 min post ejaculation. The latency to satiation and the number ejaculations are recorded.

Finally, for each ejaculatory series, the following parameters are recorded:

- **Copulation duration:** time interval between the first mount and the ejaculation.
- **Post ejaculatory interval:** time interval from the ejaculation to the first mount of the next series.

Penile erections

One hour before the experiment, male rats are allowed to adapt to the quiet observation room. One hour after oral drug administration, rats are placed in individual plastic cages (10.5 × 24 × 16 cm). Series of nine rats comprising, at random, control and drug-treated animals, are observed simultaneously for 45 min and the number of penile erections is counted. Penile erection is defined as a period of pelvic thrusting followed by an upright position with genital grooming and the display of the engorged penis. Animals are used only once.

EVALUATION

Data are analyzed using non-parametric statistics. The Fisher test is used for percentage responding. For quantified behavioral parameters, the Kruskal-Wallis test, followed by the Mann-Whitney *U*-test corrected by Holm's method is used for comparisons versus the control group. The Mann-Whitney *U*-test is used for comparison of a single treated group versus its own control group.

MODIFICATIONS OF THE METHOD

Pomerantz et al. (1993) studied the influence of 5-HT receptor agonists on male sexual behavior of **rhesus monkeys**.

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E.7 Anti-Parkinsonism activity

E.7.0.1 General considerations

According to our present knowledge the fundamental lesion in Parkinson's disease is a marked deficiency in the dopaminergic innervation of the basal ganglia owing to degeneration of neurones in the substantia nigra. Enhancement of dopaminergic transmission restores al least partially motor function. The decrease in dopaminergic activity in the basal ganglia results in a relative excess of cholinergic influence. Therefore, dopaminergic agonists, such as levodopa, a precursor of dopamine, and cholinergic (muscarinic) antagonists can be combined in the treatment of Parkinson's disease. Parkinson-like syndromes also occur after depletion of central stores by reserpine and after treatment with phenothiazines and other antipsychotic drugs blocking dopamine receptors. Survey on methods being used have been given by Vernier (1964), Marsden et al. (1975), Duvoisin (1976). Parkinsonism induced by dopaminergic antagonists has been discussed by Hornykiewicz (1975) and antimuscarinic actions of neuroleptic drugs by Miller and Hiley (1975).

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E.7.1 *In vivo* methods

E.7.1.1 Tremorine and oxotremorine antagonism

PURPOSE AND RATIONALE

The muscarinic agonists tremorine and oxotremorine induce parkinsonism-like signs such as tremor, ataxia, spasticity, salivation, lacrimation and hypothermia. These signs are antagonized by anticholinergic drugs.

PROCEDURE

Groups of 6–10 male NMRI mice weighing 18–22 g are used. They are dosed orally with the test compound or the standard (5 mg/kg benzatropine mesilate) 1 h prior the administration of 0.5 mg/kg oxotremorine s.c. Rectal temperature is measured before administration of the compound (basal value) and 1, 2 and 3 h after oxotremorine injection. Tremor is scored after oxotremorine dosage in 10 s observation periods every 15 min for 1 h.

Tremor	*Score*
absent	0
slight	1
medium	2
severe	3

Salivation and lacrimation are scored 15 and 30 min after oxotremorine injection.

absent	0
slight	1
medium	2
severe	3

EVALUATION

Hypothermia

The differences of body temperature after 1, 2 and 3 h versus basal values are summarized for each animal in the control group and the test groups. The average values are compared statistically.

Tremor

The scores for all animals in each group at the 3 observation periods are summarized. The numbers in the treated groups are expressed as percentage of the number of the control group.

Salivation and lacrimation

The scores for both symptoms for all animals in each group are summarized at the 2 observation periods. The numbers in the treated groups are expressed as percentage of the number of the control group.

CRITICAL ASSESSMENT OF THE METHOD

The oxotremorine antagonism has been proven to be a reliable method for testing central anticholinergic activity. The overt isomorphism between the animal model and the symptoms of Parkinson's disease recommend this test for screening of anti-Parkinson drugs. However, the model measures only central anticholinergic activity (Duvoisin 1976).

MODIFICATIONS OF THE METHOD

Matthews and Chiou (1979) developed a method for quantifying resting tremors in a rat model of limb dyskinesias. The model involved permanent cannulation of the caudate nucleus for the introduction of carbachol. Tremors were quantified with a small transducer and an electronic data collecting system. The system allows the construction of dose-response curves for tremor inhibition by potential antiparkinsonism drugs.

Johnson et al. (1986) developed a procedure for quantifying whole-body tremors in mice. Displacement of a free floating platform by animal movement created a change in resistance across a strain gauge. Administration of oxotremorine, 2.5 mg/kg, i.p, produced numerous high-frequency, high-intensity peaks within 5 min.

Clement and Dyck (1989) constructed and tested a tremor monitor that quantitates soman- and oxotremorine-induced tremors. The device consisted of a force transducer, from which a plastic beaker was suspended containing a mouse. The signal from the force transducer was fed into a tremor monitor and quantitated using the Applecounter from Columbus Instruments.

Coward et al. (1977) recommended N-carbamoyl-2-(2,6-dichlorophenyl)acetamidine hydrochloride (LON-954), a tremorigenic agent, as alternative to oxotremorine for the detection of anti-Parkinson drugs.

Rats treated with 3-acetylpyridine show a selective degeneration of neurons in the inferior olive nucleus and the olivo-cerebellar tract with characteristic motor incoordination and ataxia (Denk et al. 1968), Watanabe et al. 1997), Kinoshita et al. 1998). Similar motor dysfunction is seen in patients with olivo-ponto-cerebellar atrophy. To measure the effect of 3-acetylpyridine and the ameliorating effect of TRH agonists in rats the maximal height of vertical jump after stimulation by a foot shock was measured.

Stanford an Fowler (1997) used a special technique for measuring forelimb tremor in rats induced by low doses of physostigmine. The rats pressed a force-sensing operandum while a computer measured force output and performed Fourier analyses on resulting force-time waveforms.

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E.7.1.2 MPTP model in monkeys

PURPOSE AND RATIONALE

N-MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) has been shown to cause symptoms of Parkinson's disease in exposed individuals. When administered to primates this compound causes a partial destruction of basal ganglia and a syndrome that resembles Parkinson's disease.

PROCEDURE

Burns et al. (1983) treated 8 adult rhesus monkeys weighing 5–8 kg over a period of 5–8 days with cumulative intravenous doses of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (N-MPTP) up to 10–18 mg/kg. These animals showed a parkinsonism like disorder (akinesia, rigidity, postural tremor, flexed posture, eyelid closure, drooling) which was reversed by the administration of L-dopa. The pathological and biochemical changes produced by N-MPTP are similar to the well established changes in patients with Parkinsonism.

The N-MPTP intoxication was applied using marmosets by Nomoto et al. (1985, 1988), Temlett et al. (1989) and by Kebabian et al. (1992) to evaluate potential antiparkinson drugs.

EVALUATION

The severity of parkinsonian symptoms is rated by trained observers using a scale of 0 (normal) to 17 (maximum severity) that assesses movement (0: normal; 1: reduced; 2: sleepy), checking movements (0: present; 1: reduced; 2: absent), attention and blinking (0: normal; 1: abnormal), posture (0: normal; 1: abnormal trunk; 2: abnormal trunk and tail; 3: abnormal trunk, tail, and limbs; 4: flexed posture), balance and coordination (0: normal; 1: impaired; 2: unstable; 4: falls), reactions (0: normal; 1: reduced; 2: slow; 3: absent) and vocalizations (0: normal; 1: reduced; 2: absent).

MODIFICATIONS OF THE METHOD

Close and Elliott (1991) studied the behavioral effects of anti-Parkinsonian drugs in normal and MPTP-treated marmosets following central microinfusions.

Kebabian et al. (1992) tested a selective D_1 receptor agonist with antiparkinsonian activity in MTPT treated marmosets.

Domino and Sheng (1993) studied the relative potency of some dopamine agonists with varying selectivities for D_1 and D_2 receptors in MPTP-induced hemiparkinsonian monkeys.

Gnanalingham et al. (1995) used MTPT-treated marmosets and found differential effects with D_1 dopamine antagonists as compared with the effects in rats with unilateral 6-OGDA-induced lesions.

Doudet et al. (1993) used intravenous administration of ^{15}O-labeled water and 6-(^{18}F)-L-fluorodopa to assess abnormal striatal activity in monkeys after long-term recovery of unilateral lesions of the dopaminergic nigro-striatal system induced by MPTP. Positron emission tomography (PET) data were examined in relation to behavioral and biological parameters, such as cerebral blood flow.

Belluzzi et al. (1994) induced a hemiparkinsonian syndrome in *Macaca nemestrina* **monkeys** by unilateral infusion of MPTP into the right coronary artery.

Raz et al. (2000) recorded hand tremor and simultaneous activity of several neurons in the external and internal segments of the globus pallidus in **vervet monkeys** before and after treatment with MPTP.

Rollema et al. (1989) compared the effects of intracerebrally administered MPP^+ (1-methyl-4-phenylpyridinium) in three species (**mouse, rat** and **monkey**) by microdialysis determinations of dopamine and metabolites in the striatum.

Asin et al. (1997) tested a selective D_1 receptor agonist in **rats** previously given unilateral 6-hydroxydopamine injections and in **macaques** previously given unilateral, intracarotid infusions of MPTP.

Lange (1989, 1990) described circling behavior in old **rats** after unilateral intranigral injection of MPTP.

Fuxe et al. (1992) studied the protection against MPTP-induced degeneration of the nigrostriatal dopamine neurons in the **black mouse**.

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E.7.1.3 Reserpine antagonism

PURPOSE AND RATIONALE

Reserpine induces depletion of central catecholamine stores. The sedative effect can be observed in mice shortly after injection, followed by signs of eyelid ptosis, hypokinesia, rigidity, catatonia, and immobility. These phenomena can be antagonized by dopamine agonists.

PROCEDURE

Male NMRI mice of either sex weighing 20–25 g are used. They are injected intraperitoneally with 5 mg/kg reserpine and tested 24 h later. Thirty min prior to observation the test compounds are injected. The animals are placed singly onto the floor of a Perspex container (30×26 cm, 20 cm high), situated on a Panlab proximity sensor unit. Horizontal movements are recorded for 10 min. Moreover, rearings and grooming episodes are registered by an experienced observer.

EVALUATION

Locomotor activity and grooming scores of drug treated animals are compared with controls treated with reserpine and vehicle only by analysis of variance.

MODIFICATIONS OF THE METHOD

Rats treated with reserpine develop spontaneous orofacial dyskinesia that has features similar to tardive dyskinesia in humans (Nisewander et al. 1994). The incidence of tongue protrusions was recorded to quantify the occurrence of oral dyskinesia.

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E.7.1.4 Circling behavior in nigrostriatal lesioned rats

PURPOSE AND RATIONALE

Unilateral lesion of the dopaminergic nigrostriatal pathway in the rat by the neurotoxin 6-hydroxydopamine (6OHDA) induces hypersensitivity of the postsynaptic dopaminergic receptors in the striatum of the lesioned side. The rats rotate in a direction towards the lesioned side (ipsilateral) when an indirect acting compound such as amphetamine is administered but to the opposite direction (contralateral) when a direct acting dopamine agonist, e.g., apomorphine, or the dopamine precursor L-dopa is given. Therefore, this test can be used for the study of central dopamine function and the evaluation of dopamine antagonists and agonists, particularly the activity of novel antiparkinsonian drugs.

PROCEDURE

Male Wistar rats weighing 200–250 g at the time of surgery are used. The are housed individually in a controlled environment with free access to food and water.

The animals are anaesthetized with sodium pentobarbital. The head is placed in a stereotaxic device (DKI 900) and positioned according to the atlas of König and Klippel. After a sagittal cut is made in the skin of the skull, a 2 mm wide hole is drilled with an electrical trepan drill. Care is taken not to lesion the meninges. A 30 gauge stainless-steel cannula connected to a Hamilton syringe is aimed at the anterior zona compacta of the substantia nigra (coordinates anterior 4.1, lateral 1.0 and dorso-ventral –2.5 from instrument zero). A total of 8 µg of 6-OHDA in 4 γ/L of saline is injected at a rate of 1 γ/L/min. After the intracranial injection the wound is closed. The animal is allowed several weeks for recovery and for development of the lesion.

Specially constructed opaque plastic spheres attached to solid state programming equipment serve as test chambers. The number of full turns, either ipsilateral or contralateral to the lesion, are recorded on an automatic printout counter every 15 min for one- or two-hours test sessions.

To determine the control values for ipsilateral turning, each subject is administered 2.5 mg/kg of d-amphetamine and immediately placed in the circling chamber for 2 h. Control values for contralateral circling are determined by injecting apomorphine at 1 mg/kg s.c. and recording the rat's circling for 1 h.

Test compounds are given i.p or sc. and the animals placed into the circling chambers. Circling is recorded over a 1 h period.

EVALUATION

Percent change of drug turns from control turns is recorded. Using various doses ED_{50} values can be calculated.

MODIFICATIONS OF THE METHOD

Etemadzadeh et al. (1989) described a computerized rotometer apparatus for recording circling behavior. The digital pulses derived from the infrared photocell detector induced by the animal rotations were fed directly to a 20-megabyte microcomputer for on-line recording and were processed further to the Digital Equipment Corporation's VAX computer with the SAS software system for statistical and graphical analysis.

Hudson et al. (1993) described a 16-channel automated rotometer system for reliable measurement of turning behavior in 6-hydroxydopamine lesioned and transplanted rats. The system is preferable to more tedious methods such as videotaping and subsequent manual analysis or various other mechanical systems.

A rotometer differentiating clockwise and counterclockwise rotations with computerized evaluation is available from Technical & Scientific Equipment GmbH, D-61348 Bad Homburg, Germany.

Carey (1989) tested stimulant drugs as conditioned and unconditioned stimuli in a classical conditioning paradigm using drug-induced rotational behavior in rats with unilateral lesions of dopamine neurons.

The production of asymmetry and circling behavior following unilateral, intrastriatal administration of neuroleptics was discussed by Costall et al. (1983).

Rotational behavior produced by intranigral injections of bovine and human β-casomorphins in rats was described by Herrera-Marschitz et al. (1989).

Perese et al. (1989) created a hemiparkinsonian model in rats in which there is 6-OHDA-induced destruction of the dopaminergic nigrostriatal pathway but sparing of the dopaminergic mesolimbic pathway.

Garrett and Holtzman (1996) compared the effects of apomorphine, d-amphetamine, cocaine and caffeine on locomotor activity and rotational behavior in rats with unilateral 6-OHDA-induced lesions of the nigrostriatal tract.

McElroy and Ward (1995) reported that the high affinity and selective dopamine D_3 receptor ligand, 7-OH-DPAT, caused 6-OHDA-lesioned rats to rotate in a direction contralateral to the lesioned side similarly to the direct-acting dopamine agonist apomorphine.

Haque et al. (1996) directly infused the neurotrophins NT3 and NT4/5 intraparenchymally in close proximity to transplanted nigral tissue placed in the dopamine depleted striatum of 6-hydroxydopamine lesioned rats.

A survey on the unilateral 6-hydroxydopamine lesion model in behavioral brain research was prepared by Schwarting and Huston (1996).

Mele et al. (1997) studied alterations in striatal dopamine overflow by *in vivo* microdialysis during rotational behavior of rats induced by amphetamine, phencyclidine and MK 801.

Smith et al. (1996) reported contralateral turning in chronically cannulated rats after stimulation of glutamate receptors by unilateral intrastriatal injections of glutamate receptor agonists, such as kainate or AMPA.

Inhibition of sinistrotorsion induced in **guinea pigs** by injection of physostigmine into the right carotid artery was proposed as a method of screening central anticholinergic activity by De Jonge and Funcke (1962).

Behavioral quantification of striatal dopaminergic supersensitivity after bilateral 6-hydroxydopamine lesions in the **mouse** was reported by Mandel et al. (1992).

Worms et al. (1988), Poncelet et al. (1993) studied turning behavior in mice induced by intrastriatal injection of neuropeptides.

Fitzgerald et al. (1992) recommended the **chakragati mouse (ckr)** , a transgenic insertional mutant, which displays lateral circling, locomotor hyperactivity and hyperreactivity as a model to study aspects of neuropsychiatric disorders associated with dopaminergic abnormalities.

Emonds-Alt et al. (1995) injected 0.3 pg senktide into the striatum of **gerbils** inducing contralateral rotations which were antagonized by intraperitoneal or oral administration of a tachykinin NK_3 receptor antagonist.

Vernier and Unna (1963) tested drugs against the tremor induced by stereotactically-oriented electric lesions in the region of the subthalamus or the mesencephalic reticular formation in **monkeys**.

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E.7.1.5 Elevated body swing test

PURPOSE AND RATIONALE

Borlongan and Sanberg (1995) proposed the elevated body swing test as a measure of asymmetrical motor behavior of hemiparkinsonian animals in a drug-free state.

PROCEDURE

Male, 8-week-old Sprague Dawley rats are anesthetized with sodium pentobarbital (60 mg/kg i.p.) and mounted in a Kopf stereotaxic frame. They are lesioned by injection of 8 µg 6-hydroxydopamine in 4 µl saline containing 0.02% ascorbic acid in the left substantia nigra (AP – 5.0, ML + 1.5, DV – 8.0). The solution is injected over a 4 min period and the needle left in place for an additional 5 min before retraction.

Seven days after the lesion, behavioral testing is performed. The animal is allowed to habituate in a Plexiglas box and attain a neutral position having all 4 paws on ground. The rat is held about 2.5 cm from the base of its tail and elevated 2.5 cm above the surface on which it has been resting. A swing is recorded whenever the animal moves its head out of the vertical axis to either side. Before attempting an other swing, the animal must return to the vertical position for the next swing to be counted. Swings are counted for 60 s over four consecutive 15 s segments. The total number of swings made to each side is divided by the overall total number of swings made to both sides to get percentages of left and right swings. The criterion of biased swing is set at 70% or higher. At 30 and 45 s, 6-OHDA-lesioned rats exhibit right-biased swings of 70% or higher compared to normal rats.

EVALUATION

A two-way ANOVA is used to analyze swing behavior data across the 15 s segments.

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E.7.1.6 Skilled paw reaching in rats

PURPOSE AND RATIONALE

Montoya et al. (1990, 1991), Abrous et al. (1993, 1994), Nikkhah et al. (1993), Barnéoud et al. (1996) used the

skilled paw reaching test as a model of Parkinson's disease in the rat. The term "staircase test" mentioned in this context, has nothing to do with the staircase test described by Thiebot et al. (1973) for evaluating anxiolytic activity in rats (see E.2.4.6).

Unilateral injection of 6-OHDA into the medial forebrain bundle results in an impairment of paw reaching on both sides which can be ameliorated by drug treatment or transplantation of a nigral cell suspension.

The apparatus has been developed after earlier studies by Whishaw et al. (1986) who investigated the contributions of motor cortex, nigrostriatal dopamine and caudate-putamen to skilled forelimb use in the rat.

PROCEDURE

Apparatus

The apparatus consists of a clear Perspex chamber with a hinged lid. A narrower compartment with a central platform running along its length, creating a trough on either side, is connected to the chamber. The narrowness of the side compartment prevents rats from turning around, so they can use only their left paw for reaching into the left trough and their right paw for reaching into the right trough. A removable double staircase is inserted into the end of the box, sliding into the troughs on either side of the central platform. Each of the steps of the staircase contains a small well, and two 45 mg saccharin-flavored pellets are placed in each well.

Learning procedure

The week before the start of the training period, the rats are deprived of food and their body weight is stabilized at 85% of the weight of non-deprived rats. At the same time, they are gently manipulated and familiarized with the appetitive saccharin-flavored pellets.

The animals then begin to learn the paw reaching task. For four weeks they are placed in the test boxes once per day for 10–15 min. The number of pellets eaten during the test period indicates the rat's success in grasping and retrieving the pellets; the number of steps from which pellets have been removed provides an index of the attempts to reach the food and how far the rat can reach; the number of missed pellets remaining at the end of the test on the floor of the side compartment indicates a lack of sensorimotor coordination in grasping and retrieving the pellets. In addition to these three parameters, it is noted which forepaw the rat used for the first movement to reach the pellet on each test day. A first choice score of +1 corresponds to the paw contralateral to the lesion, a score of –1, to the paw ipsilateral to the lesion. Because rodents exhibit a "pawedness", it must be noted whether there is a preference for one paw.

Lesions

The mesotelencephalic system is lesioned by a stereotaxic unilateral injection of 6-OHDA into the medial forebrain bundle under equithesin anesthesia. 6-OHDA is injected in a volume of 1.5 µl and at a concentration of 4 µg/µl 0.9% saline and 0.01% ascorbic acid twice over 3 min via a 30-gauge stainless steel cannula at the stereotaxic coordinates: $L = 1.6$ mm, $AP = 0$ mm, $V = -7.6$ mm and $L = 1.6$ mm, $AP = -1$ mm, $V = -8$ mm. The coordinates AP and L are estimated relative to the bregma, and V is measured from the level of the dura, with the incisor bar set 5 mm above the interaural line. Following each injection, the cannula is left in place for an additional 4 min to allow the diffusion of the neurotoxin away from the injection site. The sham-operated group receives sham lesions by identical injection of ascorbate-saline solution alone.

Drug treatment

The animals are injected i. p. with the test drug or saline 30 min before the unilateral 6-OHDA lesion and 24 h thereafter.

EVALUATION

Test sessions are performed 4, 5, 7 and 8 weeks after 6-OHDA lesion. The parameters success, attempts and sensorimotor coordination are subjected to a two-way ANOVA with group as the independent measure and weeks as the dependent measure.

MODIFICATIONS OF THE METHOD

Fricker et al. (1996) investigated the effect of unilateral ibotenic acid lesions in the dorsal striatum, placed at either anterior, posterior, medial, or lateral loci, in the staircase test of skilled forelimb use.

Nakao et al. (1996) studied paw-reaching ability in rats with unilateral quinolinic acid lesions of the striatum as an animal model for Huntington's disease.

Barneoud et al. (1996) evaluated the neuroprotective effects of riluzole using impaired skilled forelimb use, circling behavior, and altered dopaminergic metabolism of the mesotelencephalic system in unilaterally 6-hydroxydopamine-lesioned rats.

Fricker et al. (1997) studied the correlation between positron emission tomography, using ligands to the D_1 and D_2 receptors, and reaching behavior in rats with ibotenic acid lesions and embryonic striatal grafts.

Grabowski et al. (1993), Marston et al. (1995) Sharkey et al. (1996) tested drug effects on skilled motor deficits produced by middle cerebral artery occlusion in rats using the paw reaching test.

Meyer et al. (1997) described a revolving food pellet test for measuring sensorimotor performance in rats.

An animal model of **Huntington's disease** was recommended by Borlongan et al. (1997). Systemic ad-

ministration of 3-nitropropionic acid, an inhibitor of the mitochondrial citric acid cycle, produces a very selective striatal degeneration and results in a progressive locomotor deterioration resembling that of Huntington's disease.

Intrastriatal injection of quinolate, a NMDA receptor agonist, replicates many neurochemical, histological, and behavioral features of Huntington's disease (Beal et al. 1986; DiFigla 1990; Pérez-Navarro 2000).

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E.7.1.7 Stepping test in rats

PURPOSE AND RATIONALE

Schallert et al. (1992), Olsson et al. (1995) introduced the stepping test as a clinically relevant unilateral model of Parkinsonian akinesia. The 6-OHDA lesion induced marked and long-lasting impairments in the initiation of stepping movements with the contralateral paw which can be ameliorated by the systemic application of drugs.

PROCEDURE

6-OHDA lesion surgery

Female Sprague Dawley rats receive two stereotaxic injections of 6-hydroxydopamine (3.6 µg/µl in 0.2 µg/ml ascorbate-saline) into the right ascending mesostriatal dopamine pathway using a 10 µl Hamilton syringe at the following coordinates (in mm, with reference to bregma and dura):

1. 2.5 µl at AP –4.4, L 1.2, V 7.8, tooth bar –2.4;
2. 3.0 µl at AP –4.0, L 0.8, V 8.0, tooth bar +3.4 at an injection rate of 1 µl/min. The cannula is left in place for an additional 5 min before slowly retracted.

Experimental setup for stepping test

The tests monitoring initiation time, stepping time and step length are performed on a wooden ramp with a length of 1 m connected to the rat's home cage. A smooth-surfaced table is used for measuring adjusted steps.

During the first 3 days the rats are handled by the experimenter to become familiar with the experimenter's grip. During the subsequent 1–2 days the rats are trained to run spontaneously up the ramp to the home cage.

The stepping test comprises two parts: first, the time to initiation a movement of each forelimb, the step length, and the time required for the rat to cover a set distance along the ramp with each forelimb; and second the initiation of adjusting steps by each forelimb when the animal was moved sideways along the bench surface. Each test consists of two tests per day for 3 consecutive days and the mean of 6 subtests is calculated.

Initiation time, stepping time, and step length

The rat is held by the experimenter with one hand fixing the hindlimbs and slightly raising the hind part above the surface. The other hand fixes the forelimb not to be monitored. Time is measured until the rat initiates movement with the forelimb not fixed by the experimenter, using 180 s as break-off point. Stepping time is measured from initiation of movement until the rat reaches the home cage. Step length is calculated by dividing the length of the ramp by the number of steps required for the rat to run up the ramp. The sequence of testing is right paw testing followed by left paw testing, repeated twice.

Adjusting steps

The rat is held in the same position as described above with one paw touching the table, and is then moved slowly sideways (5 s for 0.9 m) by the experimenter, first in the forehand and then in the backhand direction. The number of adjusting steps is counted for both paws in the backhand and forehand direction of movement. The sequence of testing is right paw forehand and backhand adjusting stepping, followed by left paw forehand and backhand directions. The test is repeated twice each day.

The paw contralateral to the lesion is passively dragging when the rat is moved in the forehand direction, while the ipsilateral paw performs frequent stepping movements.

Drug application

Stepping tests are repeated as baseline weekly after the 6-OHDA lesion. The drug tests are administered during 1 day only. Various drugs can be evaluated in weekly intervals.

EVALUATION

Results are expressed as means SEM. For statistical evaluation, the data are subjected to one-factor analysis of variance (ANOVA) and Fisher post hoc test.

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E.8 General anesthesia

E.8.1 Intravenous anesthesia

E.8.1.1 General considerations

PURPOSE AND RATIONALE

The first agents which could be used as intravenous anesthetics were **barbiturates**. Barbiturates with a duration of action appropriate to the requirements of surgery became available with the introduction of hexobarbital and thiopental (Volwiler and Tabern 1930; Miller et al. 1936). The studies with barbiturates were extended (Butler and Bush 1942; Christensen and Lee 1973). Intravenous anesthetics from other chemical groups were developed, such as **acetamidoeugenol** (Estil, Domenjoz 1959), steroid derivatives (Presuren = **hydroxydione sodium**, Laubach et al. 1955; **alfaxolone**, CT1 341, Child et al. 1971), **propanidid** (Epontol, Goldenthal 1971), **ketamine** (CI-581, Chen et al. 1966; Reich and Silvay 1989), **etomidate** (Janssen et al. 1975), **propofol** (ICI 35 868, Glenn 1980), **midazolam** (Pieri 1983; Reilly and Nimmo 1987).

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E.8.1.2 Screening of intravenous anesthetics

PURPOSE AND RATIONALE

Screening of intravenous anesthetics or hypnotics is performed mostly in mice or rats. Doses for loss of righting reflex and lethal doses are determined. Onset of action and duration of action are the secondary parameters.

PROCEDURE

Male mice weighing 18–22 g are injected intravenously via the tail vein. The anesthetic activity is estimated from the number of animals that loose their righting reflex. The righting reflex is considered lost when the mouse, placed on its back, fails to recover from this position within 1 min. The acute toxicity is based on lethality within a 24-h observation period.

To determine onset and duration of action groups of 20 mice are used. They are placed in individual observation cages maintained at room temperature (24 ±1 °C). They are not stimulated during the interval between loss and recovery of the righting reflex. The onset is defined as the complete loss of the righting reflex, i.e., no attempt to move the head or body. Recovery is considered to have occurred when the animal after spontaneous righting would re-right itself within 15 s when placed on its back.

EVALUATION

The median anesthetic dose (AD_{50}) and the median lethal dose (LD_{50}) are determined from dose-response curves with at least 4 doses by the method of Litchfield and Wilcoxon (1949).

The data for onset and duration of action are analyzed statistically by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Volwiler and Tabern (1930) determined the minimum effective dose in rats after subcutaneous injection of various barbiturates not being awakened when outer ear passage was tickled with a straw.

Büch et al. (1968) studied the distribution, anaesthetic potency and metabolic elimination of the optical isomers of methylphenobarbital in rats.

Glenn (1977) described a method for the laboratory evaluation of the speed of onset of i.v. anesthesia in **mice**. Various clinically used intravenous anesthetics were compared. The technique involves a) determination of the medium hypnotic dose (HD_{50}) by plotting the probit value of the mice sleeping against dose on a logarithmic scale, b) plotting mean induction time over a range of doses against the logarithm of the dose and c) comparison of induction times at 1.25 HD_{50}. All doses were given over 1 s or 10 s. A 1-s injection was thought to be of most value in the of structure activity effects.

Chen et al. (1966) tested the anesthetic activity and the neuropharmacological spectrum of ketamine (CI-581) in mice, pigeons and monkeys.

Child et al. (1971) tested the anesthetic activity of alfaxolone (CT1 341) in **mice, rats, rabbits, cats, dogs** and **monkeys**.

Janssen et al. (1975) tested onset and duration of anesthesia after etomidate in mice, rats, guinea pigs and dogs.

New intravenous anesthetics were reviewed by Reilly and Nimmo (1987).

The anesthetic potency of remifentanil in dogs in terms of reduction of enflurane *MAC* was tested by Michelsen et al. (1996).

Dingwall et al. (1993) described the tolerometer as a fast, automated method for the measurement of righting reflex latency.

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E.8.1.3 EEG threshold test in rats

PURPOSE AND RATIONALE

The electroencephalographic (EEG) threshold test has been used to determine and compare the potency of several CNS depressant agents (Bolander et al. 1984; Koskela and Wahlström 1989; Norberg and Wahlström 1988; Norberg et al. 1987). Korkmaz and Wahlström (1997) described in detail the protocol of the EEG burst suppression threshold test for the determination of CNS sensitivity to intravenous anesthetics in rats.

PROCEDURE

Adult Sprague-Dawley rats are housed at a reversed light/dark cycle and an ambient temperature of 23 ±1 °C. Twenty four hours prior to the EEG threshold test, the rats are placed in a tube restrainer. Twisted stainless steel wire and suitable surgical needles are used to sew the electrodes to the scalp above the frontal cortex. Since generalized changes in EEG recordings are used, this stainless steel material is adequate for recording purposes. Care is taken to prevent irritation of periosteal tissue. Since this procedure causes little discomfort, the use of local anesthetics and general anesthesia can be avoided.

For EEG threshold testing, the rat is placed on a warm cloth and held gently by the assistant. A needle is placed on one lateral tail vein and connected with an infusion pump. Crocodile clips are used to connect the electrodes to the EEG recorder and a crocodile clip is attached to one of the ears of the rats as a signal ground.

The EEG recording is closely observed by the technician. The changes in the EEG induced by the anesthetic agent are used to measure drug effects on the CNS. The normal EEG in an awake rat has a low amplitude and a frequency of approx. 30 cycles/s. During the first part of infusion, an increase in amplitude and a slight decrease in frequency are observed. At this stage of infusion, dependent on the anesthetic agent, jerks or sometimes convulsive episodes may occur. As the infusion continues, the frequency decreases and burst suppression periods appear. The loss of righting reflex occurs at this stage. When a burst suppression lasts one second or more, the threshold criterion which is called 'silent second' is reached and the time is recorded. After the threshold determination, the rats are placed in the recovery room.

EVALUATION

The threshold dose is calculated by multiplying the time required to reach the threshold criterion with the dose administration rate. Threshold doses are determined for each anesthetic at various dose administration rates indicating the optimal dose administration rate.

MODIFICATIONS OF THE METHOD

Wauquier et al. (1988) studied relationships between quantitative EEG measures and pharmacodynamics of alfentanil in dogs. Before, during and up to 3 h after infusion, the effects of 3 doses on 6 quantitative EEG measures (zero-crossing frequency, root mean square amplitude, spectral edge, relative delta, alpha and beta power) were assessed.

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E.8.1.4 Efficacy and safety of intravenous anesthetics

PURPOSE AND RATIONALE

Besides determination of the ratio between anesthetic and lethal dose, intravenous anesthetics have to be tested for their influence on the cardiovascular and pulmonary system. Borkowski et al. (1990 described a method to compare intravenous anesthetics in rabbits.

PROCEDURE

Adult New Zealand White rabbits with a mean weight of 4.5 kg are used. To provide access for direct blood pressure measurement and arterial blood samples, an 18-gauge catheter is implanted into the left carotid artery under halothane anesthesia. Following a minimum 24-h recovery period, the rabbit is placed in a sling and a pneumograph is fitted around the rabbit's caudal thorax at the level of 10^{th} to 12^{th} ribs to monitor respiratory rate and pattern. From the arterial catheter blood is withdrawn for blood gas analysis. Then the catheter is connected to a blood-pressure transducer. A 10-min acclimatization period is allowed before control measurements are recorded. Each rabbit serves as its own control in that cardiopulmonary parameters and responses to noxious stimuli are determined before anesthesia is induced. The right marginal ear vein is catheterized with a 22-gauge catheter, which is secured with adhesive tape, flushed with physiologic sterile saline and used for the administration of the anesthetic agents.

One third of the dose of the anesthetic to be tested is injected manually over a 1-min period. When the rabbit is relaxed it is removed from the sling and is placed in left lateral recumbence on a heating blanket. The degree of muscle tension and reaction to noxious stimuli are determined while the rabbit is in the sling and at 15 min intervals following anesthesia. The assessments performed include those of jaw tone, leg muscle tone, palpebral reflex, corneal reflex, ear pinch reflex and pedal withdrawal reflex. Jaw tone is evaluated subjectively by pulling the lower jaw open by an index finger. Leg muscle tone is evaluated by flexion and extension of the right rear leg according to subjective scores. The corneal reflex is tested by placing a moistened cotton swab on the cornea. The palpebral reflex is tested by touching the medial canthus with a dry cotton swab. Assessment of the ear pinch reflex is performed by applying a compression force with an alligator clip. The pedal withdrawal reflex is determined by applying the same clip on the right rear fifth digit at the distal phalanx.

Cardiopulmonary parameters and rectal body temperature are determined while the rabbit is in the sling and also at 15 min intervals following induction of anesthesia with the rabbit in lateral recumbency. Heart rate, mean arterial blood pressure, respiratory rate and respiratory pattern are calculated from tracings from the physiological recorder. Arterial blood pH, partial pressure of oxygen (PaO_2), and partial pressure of carbon dioxide ($PaCO_2$) are determined from arterial blood samples.

EVALUATION

The heart rate, mean arterial blood pressure, respiratory rate, pH, PaO_2 and $PaCO_2$ are analyzed using a two-factor analysis on repeated measures. The control values are treated as covariate to allow standardization of the inherent variation between rabbits. The single *t*-test for paired differences is used to compare control values to data obtained during the later testing intervals. The standard error of the mean (SEM) is calculated for each variable at each time interval. Data for muscle tone and responses to noxious stimuli are calculated as frequency percentages. The Fisher's Exact test is used to compare between treatments. For all of the statistical analyses, a *p*-value of less than 0.05 is considered significant.

MODIFICATIONS OF THE METHOD

Details of anesthesia in the rabbit were also described by Murdock (1969).

Peeters et al. (1988) performed a comparative study of four methods for general anesthesia in rabbits.

Glenn (1980) examined the anesthetic activity of propofol (ICI 35 868) in mice, rats, rabbits, cats pigs and monkeys including cardiovascular and respiratory parameters and EEG studies.

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E.8.2 Inhalation anesthesia

E.8.2.1 General considerations

PURPOSE AND RATIONALE

The efficacy and safety of new inhalation anesthetics has to be evaluated in pharmacological experiments.

Robbins (1946) defined the anesthetic AD_{50} as the concentration of anesthetic at which 50% of mice failed to right themselves for 15 s when placed in a rotating bottle with a known concentration of anesthetic. The concentration of the anesthetic that caused apnea in 50% of the mice in 10 min was defined as the LD_{50} and the ratio LD_{50}/AD_{50} as and an index of safety.

The term **"minimum alveolar anesthetic concentration" (MAC)** was coined by Merkel and Eger (1963) as an index to compare various anesthetic agents.

For man, Saidman and Eger (1964) defined *MAC* as the point at which 50% of the patients moved in response to a surgical incision.

The use of *MAC* which represents the partial anesthetic pressure in the brain has gained wide acceptance (Eger et al. 1965; Quasha et al. 1980).

A method for determining minimum alveolar concentration of anesthetic in the rat was published by Waizer et al. (1973).

Kashimoto et al. (1997) determined the minimum alveolar concentration of sevoflurane in rats.

Seifen et al. (1987) used *MAC* values for comparison of cardiac effects of enflurane, isoflurane, and halothane in the dog heart-lung preparation.

Determination of an anesthetic index (Apnea/*MAC*) in experiments in dogs has been proposed by Regan and Eger (1967).

Determination of the minimal alveolar concentration (*MAC*) of halothane in the white New Zealand rabbit was published by Davis et al. (1975).

Wolfson et al. (1972) recommended brain anesthetic concentration for construction of anesthetic indices.

Murphy and Hug (1982), Hall et al. (1987) used the reduction of enflurane *MAC* values in dogs as parameter for the anesthetic potency of fentanyl or sufentanyl, respectively.

Eger et al. (1988) determined minimum alveolar concentration of fluorinated anesthetics in pigs.

Fang et al. (1997) found that maturation decreases ethanol minimum alveolar anesthetic concentration (*MAC*) more than desflurane *MAC* in rats.

Ide et al. (1998) used airway occlusion in cats as a noxious respiratory stimulus that induces a visceral sensation of choking for determination of minimum alveolar anesthetic concentrations during halothane, isoflurane, and sevoflurane anesthesia. These values were compared with *MAC* values for somatic noxious stimuli such as toe pinch or tetanic stimulus. The authors recommended this method as a new concept for *MAC* determination.

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E.8.2.2 Screening of volatile anesthetics

PURPOSE AND RATIONALE

A simple technique for preliminary testing of anesthetic agents is the introduction of a measured amount of volatile liquid into a covered glass container of known volume. If the density and molecular weight of the liquid are known, the volume % concentration of the anesthetic mixture can be calculated (assuming 1 mol of vapor = 22.4 l). Mice or rats are introduced into the chamber and the quality of anesthesia is noted. Conditions are then adjusted until the anesthetic concentration has been established.

PROCEDURE

Male NMRI mice weighing 20–25 g or male Wistar rats weighing 250–300 g are used. A wide-mouth, screw-cap glass jar of 3 Liter volume is flushed with oxygen for 1 min and a measured amount of the volatile substance is placed on the bottom through a suitable syringe. The amount is calculated to give 1.25 vol% concentration of vapor in the jar (or a logarithmic multiple of 1.25%, i.e., 0.63, 2.5, 5.0, 10.0). The jar is closed and evaporation of the substance is facilitated by gentle rotation of the jar. One rat or 5 mice are quickly placed from a beaker into the jar, and the jar is immediately closed. Every 15 s the jar is gently rotated and the time noted for each animal to become anesthetized (loss of righting reflexes). The procedure is repeated until all animals are anesthetized. Induction should occur not sooner than 30 s and not later than 5 min. The animals are allowed to remain in the anesthesia jar for 10 min, with testing of righting reflexes until they are quickly removed into room air. The time of recovery to righting or walking is recorded for each animal. Postanesthetic analgesia is tested by gently pressing the base of the tail every min until recovery has occurred. If induction time is shorter than 30 s or longer than 10 min, the concentration of anesthetic is decreased or increased until the proper concentrations is found.

EVALUATION

The results are reported as mean induction time and mean recovery time. Twenty-four-hour survival rate is recorded for latent toxicity.

MODIFICATIONS OF THE METHOD

Burns et al. (1961) used a simplified mouse test apparatus with a small container and an open circuit technique.

Raventós (1956) used cats, dogs and monkeys to evaluate the cardiovascular effects of fluothane.

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E.8.2.3 Efficacy and safety of inhalation anesthetics

PURPOSE AND RATIONALE

To assess the safety margin of a inhalation anesthetic not only the ED_{50} values but also the maximally effective dose and the dose with a minimal danger of fatal outcome should be determined. In particular, *cardiovascular parameters* are observed.

PROCEDURE

Male Sprague Dawley rats weighing 300–350 g are placed into a clear chamber with the tail protruding from a special opening. An anesthetic-oxygen nonhumidified mixture is directed into the chamber at a rate of 4 l/min. The inhalation anesthetics, e.g., halothane or isoflurane, are vaporized in Draeger® vaporizers and the level in the chamber is monitored with a gas analyzer which is calibrated with a mass spectrometer. Rectal temperature is monitored and maintained at 37 °C with a heating pad. Each rat is exposed to only one pre-determined concentration of anesthetic for 30 min, at which time the presence or absence of the end point of anesthesia is determined. For the lethal end point, rats are tracheotomized and ventilated at 60 strokes/min through an endotracheal catheter. Tidal volume is adjusted to maintain Pa_{CO_2} at 40 ±5 mm Hg.

As endpoints of anesthesia are used:

- Loss of righting reflex. The test is regarded as positive if the animal fails to right itself with all four feet on the floor within 15 s after being placed in a side position.
- Prevention of purposeful movements response to a noxious stimulus. The animals are stimulated for 60 s by placement of a 1-kg weight on the middle of the tail. Only the purposeful movement of the head or legs is considered to be a response.
- Prevention of the heart rate increase to a noxious stimulus (ECG signals). An increase in heart rate of greater than 1% is regarded as a positive response.
- The end point for the lethal effect is 7 mm Hg in the femoral artery with artificial respiration.

With each of the anesthetics, four series of experiments are performed: to determine the righting reflex, purposeful movement response, heart rate response, and lethal effect. The concentrations of the test compounds and the standard are spaced equally between the abovementioned doses.

After determination of the heart rate effect and the lethal effect, the rats are sacrificed for determinations of brain tissue concentrations. The whole brain is re-

moved and tissue anesthetic concentration is determined by gas chromatography.

EVALUATION

For calculation of the dose-effect curves, the probit method of statistical analysis is used.

For the assessment of anesthetic safety, not only the therapeutic ratio (LD_{50}/ED_{50}) but also the standard safety margin

$$SSM = (LD_5 - ED_{95}) / ED_{95} \times 100$$

is used. This represents the percentage by which the ED_{95} has to be increased before LD_5 is reached.

CRITICAL ASSESSMENT OF THE METHOD

The standard safety margin has definitive advantages over therapeutic ratio. In contrast to the LD_{50}/ED_{50} index, the standard safety margin is influenced not only by the distance between central points of the anesthetic and lethal dose-effect curves, but also by the slope of these curves.

MODIFICATIONS OF THE METHOD

A similar concept, based on response to tail clamping, respiratory arrest and cardiovascular failure in the *rat* was published as anesthetic index by Wolfson et al. (1973).

An other attempt to determine anesthetic requirements in rats was published by White et al. (1974).

Kissin et al. (1984) studied the morphine-halothane interaction in rats.

Fukuda et al. (1996) investigated the effects of sevoflurane and isoflurane on bupivacaine-induced arrhythmias and seizures in rats.

Krantz et al. (1941, 1953) described an anesthetic index between surgical anesthesia (cornea and wink reflexes abolished) and respiratory failure in **dogs**.

Van Poznak and Artusio (1960a,b) determined the anesthetic properties of fluorinated compounds in dogs using a face mask for the induction of anesthesia and a cuffed endotracheal tube later on. ECG (lead II) and EEG were monitored.

Steffey and Howland (1978) determined the potency of enflurane in dogs in comparison with halothane and isoflurane.

Johnson et al. (1998) compared isoflurane with sevoflurane for anesthesia induction and recovery in adult dogs.

Salmempera et al. (1992) studied in dogs the potency of remifentanil, a short acting opioid analgesic, which is used as anesthetic adjunct by variable-rate infusion. Enfluorane minimal alveolar concentration was measured by the tail-clamp method in dogs before and after sequential infusion of various doses of remifentanil. The plasma concentration causing a 50% reduction of enfluorane minimal alveolar concentration was determined.

Kataoka et al. (1994) studied the negative inotropic effects of sevoflurane, isoflurane, enflurane and halothane in canine blood-perfused papillary muscles.

Hirano et al. (1995) compared the coronary hemodynamics during isoflurane and sevoflurane anesthesia in dogs.

Mutoh et al. (1997) compared the cardiopulmonary effects of sevoflurane with those of halothane, enflurane, and isoflurane, in dogs.

Hashimoto et al. (1994) examined the effects of sevoflurane and halothane on the effective refractory period and ventricular activation in a canine myocardial infarction model.

The effects of desflurane, sevoflurane and halothane on postinfarction spontaneous dysrhythmias in dogs were examined by Novalija et al. (1998).

Cardiopulmonary effects in **cats** were studied for desflurane by McMurphy and Hodgson (1996), for sevoflurane by Hisaka et al. (1997).

Saeki et al. (1996) determined the effects of sevoflurane, enflurane, and isoflurane on baroreceptor-sympathetic reflex in **rabbits**.

Hanagata et al. (1995) found that isoflurane and sevoflurane produce a dose-dependent reduction in the shivering threshold in rabbits.

The effects of multiple administrations of sevoflurane to cynomolgus **monkeys** were evaluated by Soma et al. (1995).

The effect of inhalation anesthetics on the **respiratory system** was investigated in several studies:

Mazzeo et al. (1996) compared the relaxing effects of desflurane and halothane at various *MAC*s on isolated proximal and distal airways of dogs precontracted with acetylcholine.

Hashimoto et al. (1996) compared the bronchodilating effect of sevoflurane, enflurane and halothane in dogs using a superfine fiberoptic bronchoscope. The dogs were anesthetized with pentobarbital, paralyzed with pancuronium, and the lungs were mechanically ventilated. The endotracheal tube had an additional lumen to insert the superfine fiberoptic bronchoscope (outer diameter 2.2 mm) which was located between a second and third bronchial bifurcation to continuously monitor the bronchial cross-sectional area of third or forth generation bronchi. Bronchoconstriction was produced by histamine injection and infusion. The bronchial cross-sectional area was printed out by a videoprinter at the end of expiration and was calculated on a computer using an image program after various *MAC*s of the different inhalation anesthetics.

Mitsuhata et al. (1994) induced systemic anaphylaxis in dogs sensitized to Ascaris suum by intravenous

injection of the antigen and measured pulmonary resistance and dynamic pulmonary compliance. Sevoflurane was as effective as isoflurane in attenuating bronchoconstriction associated with anaphylaxis in dogs.

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Chapter F
Drug effects on learning and memory[1]

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F.1 Introduction

It is easily understood that behavioral psychopharmacologies faced with the task of dealing with extremely complex behavioral disturbances. This holds true for both patient groups: young people with learning and memory problems and elderly patients with memory deficits. For the elderly, difficulties arise in designing appropriate animal models of human aging or the deficits occurring during human aging. One of the major

problems for experimental behavioral pharmacology is whether or not old animals are the appropriate models. At the first view it seems obvious that the study of potential geronto-psychopharmacologic drugs should be performed in old animals. However, the problem is much more complicated. Laboratory animals are not a homogenous population, especially when old. Most of these old animals who are one third survivors of a population have an individually different pathological history which is mostly unknown to the investigators. Some animals may be arthritic others may have bronchitis or cardiac deficiencies. If, for example, an arthritic rat is given a performance task associated with lever pressing, the animals may fail because of his rigid and painful joints and not because of a brain deficit or of the ineffectiveness of the test compound. Similar effects can be observed with old animals having a cataract in a visual discrimination task. Failure to perform a task may even be the result of both central and peripheral disturbances. Consequently it is impossible to describe the failure of one animal to perform the task to deficits in some parts of the brain or to pathological changes in the body.

Considerable evidence indicates that there are age-related changes in learning and memory (Gold and McGaugh 1975; Gold et al. 1975). For certain kinds of learning, there are specific developmental periods at which acquisition and retention are optimal; for example, imprinting in animals (Hess 1972; Scheich 1987) and language acquisition in humans (Lennenberg 1967). For other forms of learning and memory, acquisition and long-term retention in animals mature gradually with development (Campbell and Spear 1972). There is some evidence that aged organisms have impaired memory function. For example, in humans, performance on recent memory tasks appears to decay at shorter training-test intervals and is more susceptible to retroactive interference in elderly subjects than in young adult subjects (Craik 1977; Kubanis and Zornetzer 1971). Recent memory also appears to be deficient in aged non-human primates (Bartus et al. 1980).

Although drug discovery is based upon many factors, animal models provide a crucial part in identifying chemical compounds with potential for clinical efficacy. A number of important animal models of human disease have come into existence based upon "serendipitous" discoveries of their ability to identify chemicals with a particular set of therapeutic actions. However, in the absence of truly effective therapeutic agents for age-associated dementia to serve as standards, the development of appropriate animal models for dementia represents a serious challenge. In the case of Alzheimer's Disease or age-associated memory impairment, successful development of animal models may well depend upon our ability to accurately reproduce specific pathophysiological or etiologic factors.

It is generally agreed that an ideal animal model of human dementia would exhibit some or all the behavioral and neurological dysfunctions known to be associated with the clinical disorder, as well as the pathological processes leading to the demented condition. An early approach was the measurement of the spinal cord fixation time in rats as described by Giurgea and Mouravieff-Lesiusse (1971), Giurgea and Salama (1977).

Since cholinesterase inhibitors, such as physostigmine (Christie et al. 1981; David and Mohs 1982; Thal et al. 1983) and tacrine (Summers et al. 1986) have been shown to improve cognitive function in patients with Alzheimer's type dementive disorders and based on analytical data in brains of aged subjects, the cholinergic hypothesis of geriatric memory dysfunction has been established. Numerous attempts have been made to study the influence of potential drugs on the central cholinergic system, e.g., inhibition of acetylcholinesterase activity in rat striatum *in vitro*, differentiation of this activity versus the inhibition of butyrylcholinesterase activity, *ex vivo* cholinesterase inhibition in rat striatum, release of acetylcholine from rat brain slices after electrical stimulation, binding to muscarinic cholinergic receptors in rat forebrain, stimulation of phosphatidyl-inositol turnover via muscarinic receptors, binding to nicotinic cholinergic receptors in rat frontal cortex. Presynaptic cholinergic markers include choline acetyl-transferase and acetylcholinesterase activities, high-affinity transport of choline, acetylcholine synthesis, and muscarinic receptor binding. The high-affinity transport of choline is influenced by a cholinotoxin, ethylcholine aziridinium (AF64A) which induces *in vivo* a persistent central cholinergic hypofunction of presynaptic origin (Fisher and Hanin 1986).

Changeux et al. (1998) discussed the role of nicotinic acetylcholine receptors in the brain in learning and reinforcement.

These approaches may give hints for the discovery of new antidementic drugs. The "cholinergic hypothesis" (Bartus et al. 1982, 1987) is not unequivocal in the pathophysiology of Alzheimer's disease. The role of other transmitters has to be studied (Nordberg 1990). *In vitro* and *in vivo* studies have to be performed in parallel in order to find new approaches to treat Alzheimer's disease or Alzheimer's type dementia.

Several animal strains have been recommended to study age-dependent memory deficits, such as the **senescence-accelerated mouse (SAM)**, (Fujibayashi et al. 1994; Takeda et al. 1994, 1996, 1997; Maurice

et al. 1996; Nishiyama et al. 1997; Flood and Morley 1998; Markowska et al. 1998),

transgenic mice which overexpress S100β, a calcium binding astrocytic protein influencing hippocampal long-term potentiation (LTP) and depression (LTD) (Roder et al. 1996),

transgenic Cu/Zn-SOD mice overexpressing the gene encoding copper/zinc superoxide dismutase which is also overexpressed in human Down syndrome (Gahtan et al. 1998),

a mutant mice strain exhibiting delayed Wallerian degeneration (Fox and Faden 1998),

mice with a modified β-amyloid precursor protein gene (Tremml et al. 1998),

epileptic fowl having a hereditary form of primary generalized epilepsy characterized by tonic-clonic seizures (Gervais-Fagnou and Tuckek 1996).

Several authors reported impairment of memory functions after **cerebral lesions**:

Ennaceur (1998) studied the effects of electrolytic lesions of the Medial Septum/Vertical Diagonal Band of Broca, the Globus Pallidus and the Substantia Innominata/Ventral Pallidum on the performance of **rats** in object-recognition memory and radial-maze learning tasks.

Chambers et al. (1996) proposed lesioning of the ventral hypothalamus in neonatal rats as an experimental model of schizophrenia with learning deficits.

Chen et al. (1997) observed a remarkable impairment of passive avoidance response by bilateral dorsal hippocampal lesions in rats.

Abe et al. (1998) investigated the effect of a benzodiazepine receptor partial inverse agonist on the impairment of spatial memory in basal forebrain-lesioned rats.

Ishikawa et al. (1997) reported hippocampal degeneration inducing impairment of learning in rats after neonatal administration of monosodium glutamate or oral administration of trimethyltin.

Carli et al. (1997) caused impairment of spatial learning in rats by intrahippocampal administration of scopolamine or 7-chloro-kynurenic acid.

Stancheva et al. (1993) studied in rats the effect of neonatal 6-hydroxydopamine treatment on learning and retention and on the level of biogenic monoamines in some brain structures as well as the influence of nootropic drugs.

Nitta et al. (1997) reported that the continuous infusion of anti-nerve growth factor monoclonal antibody into the septum of rats produces an impairment of memory.

Misztal et al. (1996) produced a short-term working memory deficit in rats by intraventricular infusion of quinolinic acid via ALZET osmotic minipumps for 2 weeks.

Inagawa (1994) reported impairment of spatial working memory in rats induced by intracerebroventriular injection of the cholinergic neurotoxin, ethylcholine mustard aziridinium picrylsulfonate.

Bjugstad et al. (1998) gave chronic intraventricular infusions of tumor necrosis factor alpha to rats and found symptoms resembling the AIDS dementia complex in humans, including learning and memory impairment.

Beers et al. (1995) described spatial recognition memory deficits in Lewis rats following encephalitis after intranasal inoculation with herpes simplex virus type 1.

Yu et al. (1997) reported significant impairment of learning and memory ability in **mice** after hippocampal microinfusion with colchicine.

Chapman et al. (1998) observed spatial working memory impairment in apolipoprotein E-deficient mice.

English et al. (1998) showed that infection with the LP-BM5 murine leukemia virus causes an AIDS-like syndrome – murine acquired immunodeficiency syndrome – in C57B1/6 mice and impairs spatial learning without gross motor impairment.

In several studies **monkeys** were used:

Gaffan (1994) described a model of episodic memory impairment in monkeys with fornix transection.

Harder et al. (1996) produced a specific pattern of cognitive deficits in the marmoset by fornix transection.

Alvarez et al. (1994) studied memory functions in monkeys with lesions of the hippocampal formation and adjacent cortex.

Fernandez-Ruiz et al. (1995) studied the long-term cognitive impairment in MPTP-treated rhesus monkeys.

Ye et al. (1999) evaluated a reversible acetylcholinesterase inhibitor for its ability to reverse the deficits in spatial memory produced by scopolamine in young adult monkeys and those that are naturally occurring in aged monkeys in a delayed-response task.

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F.2 *In vitro* methods

F.2.0.1 *In vitro* inhibition of acetylcholine-esterase activity in rat striatum

PURPOSE AND RATIONALE

The purpose of this assay is to screen drugs for inhibition of acetylcholine-esterase activity. Inhibitors of this enzyme may be useful for the treatment of Alzheimer's disease.

Acetylcholinesterase (AChE) which is sometimes called true or specific cholinesterase, is found in nerve cells, skeletal muscle, smooth muscle, various glands and red blood cells (Nachmansohn and Rothenberg 1945; Koelle et al. 1950; Ellman et al. 1961). AChE may be distinguished from other cholinesterases by substrate and inhibitor specificities and by regional distribution. Its distribution in brain roughly correlates with cholinergic innervation and subfractionation shows the highest level in nerve terminals.

It is generally accepted that the physiological role of AChE is the rapid hydrolysis and inactivation of acetylcholine. Inhibitors of AChE show marked cholinomimetic effects in cholinergically-innervated effector organs (Taylor 1996) and have been used therapeutically in the treatment of glaucoma, myasthenia gravis and paralytic ileus. However, recent studies (Christie et al. 1981; Summers et al. 1981; Davies and Mohs 1982; Atak et al. 1983) have suggested that AChE inhibitors may also be beneficial in the treatment of Alzheimer's dementia.

Augustinsson (1971) reviewed a number of methods for assaying cholinesterase activity and concluded that the method described by Ellman et al. (1961) was one of the best. The method described is a modification of Ellman's procedure.

PROCEDURE

Reagents

1. 0.05 M Phosphate buffer, pH 7.2
   a) 6.85 g $NaH_2PO_4 \cdot H_2O$/100 ml distilled H_2O
   b) 13.40 g $Na_2HPO_4 \cdot 7H_2O$/100 ml distilled H_2O
   c) add a) to b) until pH reaches 7.2
   d) Dilute 1 : 10
2. Substrate in buffer
   a) 198 mg acetylthiocholine chloride (10 mM)
   b) q.s. explain to 100 ml with 0.05 M NaH_2PO_4, pH 7.2 (reagent 1)
3. DTNB in buffer
   a) 19.8 mg 5,5-dithiobisnitrobenzoic acid (DTNB) (0.5 mM)
   b) q.s. to 100 ml with 0.05 M NaH_2PO_4, pH 7.2 (reagent 1)
4. A 2 mM stock solution of the test drug is made up in a suitable solvent and q.s. to volume with 0.5 mM DTNB (reagent 3). Drugs are serially diluted (1 : 10) such that the final concentration (in cuvette) is 10^{-4} M and screened for activity. If active, IC_{50} values are determined from the inhibitory activity of subsequent concentrations.

Tissue preparation

Male Wistar rats are decapitated, brains rapidly removed, corpora striata dissected free, weighed and homogenized in 19 volumes (approximately 7 mg protein/ml) of 0.05 M NaH_2PO_4, pH 7.2 using a Potter-Elvejhem homogenizer (Kontes, Vineland, NJ). A 25 µl aliquot of this suspension is added to 1 ml of the vehicle or various concentrations of the test drug and reincubated for 10 min at 37 °C.

Assay

Enzyme activity is measured with the Beckman DU-50 spectrophotometer. This method can be used for IC_{50} determinations and for measuring kinetic constants.

Reagents are added to the blank and sample cuvettes as follows:

Blank:	0.8 ml PO_4 buffer/DTNB
	0.8 ml buffer/Substrate
Control:	0.8 ml PO_4 buffer/DTNB/Enzyme
	0.8 ml PO_4 buffer/Substrate
Drug:	0.8 ml PO_4 buffer/DTNB/Drug/Enzyme
	0.8 ml PO_4 buffer/Substrate

Blank values are determined for each run to control for non-enzymatic hydrolysis of substrate and these values are automatically subtracted by the kindata program available on kinetics soft-pac module. This program (Beckman DU-50 series spectrophotometer, kinetics Soft-Pac™ module operation instructions: 1–7 also calculates the rate of absorbance change for each cuvette.

EVALUATION

For IC_{50} determinations: Substrate concentration is 10 mM diluted 1 : 2 in an assay yielding a final concentration of 5 mM. DTNB concentration is 0.5 mM yielding 0.25 mM final concentration

$$\% \text{ Inhibition} = \frac{\text{slope control} - \text{slope drug}}{\text{slope control}} \times 100$$

IC_{50} values are calculated from log-probit analysis.

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F.2.0.2 *In vitro* inhibition of butyrylcholine-esterase activity in human serum

PURPOSE AND RATIONALE

This assay can be used in conjunction with the acetylcholine-esterase assay to determine the enzyme selectivity of various cholinesterase inhibitors.

Butyrylcholine-esterase (BChE), which is sometimes called pseudocholinesterase, preferentially hydrolyzes butyrylcholine. This enzyme is found in the highest amounts in serum, but its physiological role is not known (Chemnitius et al. 1983; Walker and Mackness 1983). Ethopropazine and tetra-isopropyl pyrophosphoramide (ISO-OMPA) are selective inhibitors of butyrylcholinesterase. In an *ex vivo* experiment with the selective BChE inhibitor, ISO-OMPA, it was shown that inhibition of butyrylcholinesterase was not correlated with any significant acute cholinomimetic effects.

PROCEDURE

Reagents

1. 0.05 M phosphate buffer, pH 7.2
   a) 6.85 g $NaH_2PO_4 \cdot H_2O$/100 ml distilled H_2O
   b) 13.40 g $Na_2HPO_4 \cdot H_2O$/100 ml distilled H_2O
   c) Add (a) to (b) until pH reaches 7.2
   d) Dilute 1 : 10
2. Substrate in buffer
   a) 225.8 mg s-butyrylthiocholine chloride
   b) q.s. to 100 ml with 0.05 M phosphate buffer, pH 7.2 (reagent 1)
3. DTNB in buffer
   a) 19.8 mg 5,5-dithiobisnitrobenzoic acid (DTNB) (0.5 mM)
   b) q.s. to 100 ml with 0.05 M phosphate buffer, pH 7.2 (reagent 1)
4. A 2 mM stock solution of the test drug is made up in a suitable solvent and q.s. to volume with 0.5 mM DTNB (reagent 3). Drugs are serially diluted (1 : 10) such that determined from the inhibitory activity of subsequent concentrations.

Enzyme Preparation

A vial of lyophilized human serum (Precilip, Biodynamics, Houston, Texas) is reconstituted in 3 ml of distilled water. A 25 ml aliquot of this suspension is added to 1 ml of the vehicle or various concentrations of the test drug and pre-incubated for 10 min at 37 °C.

Assay

Enzyme activity is measured with the Beckman DU-50 spectrophotometer. This method can be used for IC_{50} determinations and for measuring kinetic constants.

Reagents are added to the blank and sample cuvettes as follows:

Blank:	0.8 ml PO_4 buffer/DTNB
	0.8 ml buffer/Substrate
Control:	0.8 ml PO_4 buffer/DTNB/Enzyme
	0.8 ml PO_4 buffer/Substrate
Drug:	0.8 ml PO_4 buffer/DTNB/Drug/Enzyme
	0.8 ml PO_4 buffer/Substrate

Blank values are determined for each run to control for non-enzymatic hydrolysis of substrate and these values are automatically subtracted by the kindata program available on kinetics soft-pac module. This program also calculates the rate of absorbance change for each cuvette.

EVALUATION

For IC_{50} determinations: Substrate concentration is 10 mM diluted 1 : 2 in assay yielding final concentration of 5 mM. DTNB concentration is 0.5 mM yielding 0.25 mM final concentration

$$\% \text{ Inhibition} = \frac{\text{slope control} - \text{slope drug}}{\text{slope control}} \times 100$$

IC_{50} values are calculated from log-probit analysis.

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F.2.0.3 *Ex vivo* cholinesterase inhibition

PURPOSE AND RATIONALE

This assay is used to determine the dose-response relationship and duration of action of cholinesterase inhibitors *in vivo.*

Cholinesterase inhibitors, including physostigmine (Christie et al. 1981; David and Mohs 1982; Thal et al. 1983) and tacrine (Summers et al. 1986) have been shown to improve cognitive functions in Alzheimer's disease. Physostigmine is a potent, but nonselective inhibitor of cholinesterase (Taylor 1996) and has a short duration of action. Tacrine also inhibits both acetylcholine-esterase (true) and butyrylcholine-esterase (pseudo), but is more potent as an inhibitor of the pseudo-enzyme (Heilbronn 1961).

The mechanism of inhibition of these two drugs is quite different. Physostigmine is a competitive inhibitor and blocks the active site of the enzyme by carbamylation of a serine hydroxyl group at the esteratic site of the enzyme (Taylor 1996; O'Brien 1969). This covalently bound carbamyl group then dissociates from the enzyme much more slowly than the acetyl group left by the natural substrate, but the inhibition is not irreversible like that of the organophosphates. The inhibition characteristics of physostigmine, i.e., submicromolar affinity for the enzyme and covalent binding of the inhibiting group, are ideal for *ex vivo* studies. Tacrine, however, is a mixed competitive inhibitor of cholinesterase (Heilbronn 1961), with lower apparent affinity than physostigmine for the enzyme (based on IC_{50} values at saturated substrate concentrations). Tacrine binds to the anionic site of cholinesterase through weak hydrophobic interactions (Steinberg et al. 1975).

Ideally, a dose-response for cholinesterase inhibition is determined first. Then a dose which gives a reasonable effect (>50% inhibition if possible) is chosen to do the time-course experiment. The effects on brain acetylcholinesterase activity are examined in striatal tissue, using 5 mM acetylthiocholine as a substrate (Ellman et al. 1961). Effects on butyrylcholine-esterase activity may be determined in plasma samples, with 5 mM butylthiocholine used as a substrate.

PROCEDURE

Reagents

1. 0.05 M Phosphate buffer, pH 7.2
   a) 6.85 g $NaH_2PO_4 \cdot H_2O$/100 ml distilled H_2O
   b) 13.40 g $Na_2HPO_4 \cdot 7\,H_2O$/100 ml distilled H_2O
   c) add a) to b) until pH reaches 7.2
   d) Dilute 1 : 10
2. DTNB in buffer
   a) 19.8 mg 5,5-dithiobisnitrobenzoic acid (DTNB) (0.5 mM)
   b) q.s. to 100 ml with 0.05 M phosphate buffer, pH 7.2 (reagent 1)
3. Substrate in buffer
   a) 198 mg acetylthiocholine chloride (10 mM)
   b) q.s. to 100 ml with 0.05 M phosphate buffer, pH 7.2 (reagent 1)

Drug treatment

Groups of four male Wistar rats are dosed i.p. or p.o. with vehicle or the test drug. For the initial dose response study, the rats are given varying doses of drug based on toxicity reported in primary overt effects testing and sacrificed at either 30 min or 1 h after dosing. The animals are observed and the occurrence of cholinergic signs is noted (piloerection, tremors, convulsions, salivation, diarrhea and chromodacryorrhea). For the time-course study, a dose of the test drug is given which gave significant inhibition of cholinesterase activity.

Tissue preparation

Male Wistar rats are decapitated, brains rapidly removed, corpora striata dissected free, weighed and ho-mogenized in 4 volumes of 0.05 M phosphate buffer, pH 7.2 using a Potter-Elvehjem homogenizer (Kontes, Vineland, NJ). A 12.5 ml aliquot of the homogenate is added to 1 ml 0.05 M phosphate buffer, pH 7.2/DTNB (reagent 2).

Assay

1. Enzyme activity is measured with the Beckman DU-50 spectrophotometer.

   Reagents are added to the blank and sample cuvettes as follows:

   Blank: 0.8 ml PO_4 buffer/DTNB (reagent 2)
   0.8 ml PO_4 buffer/Substrate (reagent 3)
   Control: 0.8 ml PO_4 buffer/DTNB/Enzyme from control animal
   0.8 ml PO_4 buffer/Substrate
   Drug: 0.8 ml PO_4 buffer/DTNB/Enzyme from treated animal
   0.8 ml PO_4 buffer/Substrate

   Blank values are determined for each run to control for non-enzymatic hydrolysis of substrate and these values are automatically subtracted by the kindata program available on kinetics soft-pac module. This program also calculates the rate of absorbance change for each cuvette.
2. Substrate concentration is 10 mM diluted 1 : 2 in the assay yielding final concentration of 5 mM. DTNB concentration is 0.5 mM yielding 0.25 mM final concentration.

EVALUATION

The percent inhibition at each dose or time is calculated by comparison with the enzyme activity of the vehicle control group.

$$\%\ \text{Inhibition} = \frac{\text{slope control} - \text{slope drug}}{\text{slope control}} \times 100$$

Ex vivo time course experiments for physostigmine

Physostigmine, 0.3 mg/kg, i.p.

Time (h)	% Inhibition striatum	Cholinergic signs
0.25	48.6	P,T,D
0.5	28.5	P,T,D
1.0	27.0	P,T
2.0	7.6	P,T
4.0	1.4	P,T

P = piloerection, *T* = tremors, *D* = diarrhea.

MODIFICATIONS OF THE METHOD

Antagonism of physostigmine-induced lethality in mice has been used by Gouret (1973) as a general indicator of central or peripheral anticholinergic activity. A low dose of physostigmine can be used for detecting procholinergic activity.

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F.2.0.4 Molecular forms of acetylcholinesterase from rat frontal cortex and striatum

PURPOSE AND RATIONALE

Different molecular forms of acetylcholinesterase can be isolated from animal tissues after solubilization in buffers containing various detergent and salt concentrations. The number of forms isolated, their relative amounts and molecular characteristics depend on the tissue source and the conditions used for solubilization of the membrane bound enzyme (McIntosh and Plummer 1973; Reiger and Vigny 1976; Trevor et al. (1976). Bon et al. (1979) have classified these forms as globular (G_1, G_2 and G_4) and asymmetric (A_4, A_8 and A_{12}), where the subscripts indicate the number of catalytic subunits. The G_1 and G_4 forms, having sedimentation coefficients of approximately 4S and 10S, respectively, are the major forms contained in bovine caudate nucleus (Grassi et al. 1982). Under conditions of high salt concentrations and detergent, AChE is quantitatively extracted from rat brain, with 10S form being the predominant component (Reiger and Vigny 1976). After solubilization, the molecular forms may be separated according to sedimentation properties by density gradient centrifugation, molecular weight by gel filtration or by electrophoretic mobility.

Although most of these studies on molecular forms of AChE have focused on the physical differences, Chan et al. (1972) reported some difference in sensitivity of the low molecular weight form to physostigmine and fluoride ion, while Lenz and Maxwell (1981) showed differential sensitivity to soman of forms separated by isoelectric focusing. Studies showing selective increases in the 10S form during development (Muller et al. 1985) and selective loss in Alzheimer's disease (Atack et al. 1983) suggest that this form of the enzyme may be developmentally and functionally more important. Cortical and striatal areas show different patterns of cholinergic innervation; the cortex having primarily extrinsic innervation, while the striatal cholinergic pathways are predominantly intrinsic (Ceullo and Sofroniew 1984).

The purpose of this procedure is to determine the effects of various cholinesterase inhibitors on the two major molecular forms of acetylcholinesterase isolated from rat striatum and cerebral cortex.

PROCEDURE

The procedure is divided into three main parts:

I. Preparation and isolation of molecular forms of AChE,
II. Assays for the marker enzymes,
III. Enzyme inhibition studies.

I.a Preparation of molecular forms of AChE

Male Wistar rats (200–250 g) are sacrificed, their brains rapidly removed and frontal cortices or corpora striata removed. The brain areas are weighed and homogenized in 5 volumes (wt/vol) of 10 mM phosphate buffer, pH 7.1, containing 1 M NaCl and 1% Triton X-100, except were indicated. The homogenates are centrifuged at 20 000 *g* for 20 min at 4 °C. The supernatant is aspirated and marker enzymes for 16S (*E. coli* β-galactosidase), 11.3S (bovine catalase) and 4.8S (horse liver alcohol dehydrogenase) fractions are added. The supernatant is then centrifuged at 37 000 rpm (140 000 *g* max) for 17.5 h in a Beckman L5-65 ultracentrifuge with a SW-60 rotor. 15-drop fractions are collected for each centrifuge tube and assayed for protein, β-galactosidase, catalase, alcohol dehydrogenase and acetylcholinesterase activity. In addition, butyrylcholinesterase can be measured.

A 400 µl sample of the 20 000 *g*-supernatant is carefully layered on top of a 5–20% continuous sucrose gradient. This gradient is made up in a centrifuge tube from 1.65 ml of 20% sucrose and 1.65 ml of 5% sucrose in homogenizing buffer by means of a gradient maker. A 50% sucrose cushion (0.5 ml) is placed at the bottom of the tube.

Fractions are collected from the bottom of the tube, i.e, the densest fractions are collected first. Each fraction is 15 drops or approximately 24 fractions are collected per centrifuge tube.

I.b Analysis of fractions

β-Galactosidase, catalase and alcohol dehydrogenase are determined by enzymic activity (Hestrin et al. 1955; Chance and Maehly 1955; Bonnichsen and Brink 1955). Protein concentrations are determined by the method of Lowry et al. (1951). Acetylcholinesterase activity or butyrylcholinesterase activity are determined by a modification of the method of Ellman et al. (1961). Briefly, 10 µl aliquots of the fractions are added to 0.25 mM dithionitrobenzoic acid (DTNB) and 5 mM acetylthiocholine or 5 mM butyrylthiocholine in 0.05 M phosphate buffer, pH 7.2 and the absorbance is measured at 412 nm. Fractions of peak acetylcholinesterase activity are characterized by their sedimentation characteristics relative to the marker enzymes and peak fractions are pooled for enzyme inhibition studies or determination of kinetic constants.

II. Assays for marker enzymes

A Equine liver alcohol dehydrogenase (ADH), sedimentation coefficient 4.8 S

1. Enzyme: alcohol dehydrogenase from equine liver, crystallized and lyophilized (Sigma Chem. Co.)
2. Reagents:
   a) β-nicotinamide adenine dinucleotide (NAD) (Sigma Chem. Co.)
   b) 0.1 M glycine-NaOH buffer, pH 9.6
   c) absolute ethanol
   d) buffer-substrate-NAD mixture:
   875 µl NAD + 875 µl ethanol + 18.75 ml 0.1 M glycine-NaOH buffer, pH 9.6
3. Assay
   10 µl enzyme fraction and
   850 µl mixture (reagent 2d)
   are incubated for 5 min at room temperature. The reaction is stopped by adding:
   300 µl 1.5 M $ZnSO_4$
   Absorbance is read at 340 nm and enzyme units are determined from a standard curve using values of 1.25, 2.5, 5, 10 and 20 mU of ADH.

B Bovine liver catalase, sedimentation coefficient 11.3 S

1. Enzyme: catalase from bovine liver, purified powder (Sigma Chem. Co., C-10)
2. Reagents:
   a) 30% hydrogen peroxide
   b) 0.05 M sodium phosphate buffer, pH 7.0
   c) mixture: 111 µl 30% hydrogen peroxide + 100 ml buffer, yielding 0.033% peroxide.
3. Assay
   10 µl enzyme fraction
   2 990 µl peroxide-buffer mixture (reagent 2c)
   Wavelength is set to 240 nm, absorbance is adjusted to 0.480 units. The amount of time is recorded for absorbance to decrease from 0.450 to 0.400. This corresponds to 3.45 µmol of hydrogen peroxide in 3 ml solution. Total catalase activity in 3 ml is 3.45 µMol/min.

C *E. coli* β-galactosidase, sedimentation coefficient 16.0 S

1. Enzyme: β-galactosidase from *E. coli*, grade VI, partially purified, lyophilized (Sigma Chem. Co)

2. Reagents:
   a) substrate: 15 mg/ml O-nitrophenyl-β-D-galacto-pyranoside (Sigma Chem. Co.) in water
   b) 0.6 M Na_2CO_3, pH 7.25
   c) 1 M $NaCO_3$
3. Assay
   10 µl enzyme fraction,
   150 µl 0.6 M phosphate buffer, pH 7.25, and
   50 µl O-Nitrophenyl-β-D-galactopyranoside (12 mM in assay) are incubated for 25 min at 30 °C. The reaction is stopped by adding:
   500 µl 1 M Na_2CO_3, 1.75 ml water.

   Absorbance is read at 420 nm and enzyme units are determined from a standard curve using values of 0.015, 0.030, 0.525, 0.125 and 0.250 U of β-galactosidase.

III. Enzyme inhibition studies
   For the enzyme inhibition studies, 25 µl aliquots of the enzyme preparation are preincubated with varying concentrations of the inhibitor for 10 min at 25 °C and acetylcholinesterase activity is determined as previously described.

EVALUATION

Values for the IC_{50} are determined by log-probit analysis of the inhibition data using six to seven concentrations of the inhibitor and represent the means of 3 separate experiments.

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F.2.0.5 Release of [^{3}H]ACh and other transmitters from rat brain slices

PURPOSE AND RATIONALE

Electrically stimulated release of [^{3}H]ACh is used as a biochemical screen for agents which may possibly enhance or inhibit release of [^{3}H]ACh through a direct muscarinic interaction or other indirect interactions.

Muscarinic autoreceptors have been shown to have a role in the regulation of ACh release in several areas of the CNS (Hadhazy and Szerb 1977; DeBelleroche and Gardiner 1982; Strittmatter et al. 1982; James and Cubeddu 1984). Direct stimulation of muscarinic receptors with muscarinic agonists or indirect stimulation with acetylcholinesterase inhibitors decreases ACh release evoked by either increased potassium concentration or electrical stimulation. Muscarinic antagonists can either block their inhibition, or, under certain conditions, enhance ACh release (James and Cubeddu 1987; Sethy et al. 1988). Furthermore, other neurotransmitters, most notably 5-HT and DA, can also inhibit [^{3}H]ACh release via interaction with 5-T_2 and D_2 heteroreceptors (Robinson 1983; Jackson et al. 1988; Muramatsu et al. 1988; Drukarch et al. 1989) and this inhibition can be reversed by the appropriate receptor antagonists. A compound's effect on [^{3}H]ACh release may provide evidence for a wide variety of releasing activities.

The advantages of using the electrically stimulated release technique on tissue slices have been described by Zahniser et al. (1986). This technique measures only presynaptic effects of test compounds.

PROCEDURE

This assay is based on the methods described by James and Cubeddu (1984, 1987).

Reagents

1. Krebs-Henseleit bicarbonate buffer, pH 7.4 (KHBB) Make a 2 liter batch, containing the following salts:

NaCl	13.84 g	118.4 mM
KCl	0.70 g	4.7 mM
$MgSO_4 \cdot 7\,H_2O$	0.58 g	1.2 mM
KH_2PO_4	0.32 g	2.2 mM
$NaHCO_3$	4.20 g	24.9 mM
$CaCl_2$	0.28 g	1.3 mM
Prior to use, add:		
Dextrose	4.00 g	11.1 mM

   Aerate for 60 min with 95% O_2/5% CO_2 on ice and check pH.
2. [Methyl-^{3}H]-choline chloride (80–90 Ci/mmol) is obtained from New England Nuclear. The final desired concentration of [^{3}H]choline is 100 nM. Add 0.25 nmol of [^{3}H]choline to 2.5 ml KHBB.
3. For most assays, a 10 mM stock solution of the test compound is prepared in a suitable solvent and diluted such that the final concentration in the assay is 10 µM. Higher or lower concentrations may be used depending on the potency of the test compound.
4. Hemicholinium-3 or HC-3 (Sigma): Make a 10 mM stock solution in H_2O. Two milliliters of this stock are then diluted to one liter in KHBB to give a final concentration of 20 µM.

Tissue preparation

Male Wistar rats (100–150 g) are decapitated, and cortical, striatal, or hippocampal tissue removed on ice and 0.4 mm slices are prepared with a McIlwain tissue slicer. The slices are made individually, placed in cold, oxygenated buffer (10–20 ml) and incubated at 35 °C for 30 min under oxygen. After this incubation, the buffer is decanted, leaving the slices behind. Then 2.5 ml of cold oxygenated buffer is added, and enough [^{3}H]choline to bring the final concentration to 100 nM. This is then incubated and shaken for 60 min at 35 °C under oxygen. After this step, the buffer is decanted and the "loaded" slices are immediately placed on nylon mesh in the stimulation chambers.

Assay

Control buffer is pumped through the chamber for 42 min at a flow rate of 0.7 ml/min, to establish a stable baseline. Under these conditions released [^{3}H]ACh is subject to hydrolysis by acetylcholinesterase. The perfusion buffer is changed to fresh KHBB containing 20 µM HC-3. The potent choline uptake inhibitor HC-3 is included to prevent the re-uptake of any [^{3}H]choline formed from the hydrolysis of released [^{3}H]ACh. This maintains the stoichiometry of the stimulated release. The evoked release has been shown to be mostly [^{3}H]ACh rather than [^{3}H]choline, whereas spontaneous release under control, drug-free conditions is mostly [^{3}H]choline (Richardson and Szerb 1974; Szerb et al. 1977; Supavilai and Karobath 1985; Saijoh et al. 1985; Nishino et al. 1987).

Special conditions

1. Stimulation parameters are set at 2 Hz (2 ms duration) for 120 s, with 1 ms delay and voltage setting of 750 SIU (250 Ω). Agonists are more potent modulators of [^{3}H]ACh release at low stimulation frequencies (5).
2. For striatal slices, 2 µM sulpiride is present in the buffer to prevent DA inhibition of ACh release.
3. In some experiments, 5 µM methysergide is present in the buffer to prevent the serotonergic inhibition of ACh release mediated by 5-HT_2 receptors.
4. In order to determine the muscarinic regulation of [^{3}H]ACh release, 10 µM atropine can be included in some experiments.
5. In some experiments, physostigmine is added to the perfusion buffer. This causes a marked inhibition of stimulated release via feedback at pre-synaptic receptors. Under these conditions, receptor antagonists enhance [^{3}H]ACh release.

After the experiment is completed, the chambers are washed with distilled water for at least 20 min, 200 ml of 20% methanol in distilled water and again with distilled water for at least 20 min.

EVALUATION

After conversion of dpm, percent fractional release is calculated for each fraction, using a Lotus program.

Percent fractional release is defined as the amount of radiolabeled compound released divided by the amount present in the tissue. "Spontaneous Release" (SP) values are the average of the two fractions preceding and the first fraction in that range after the stimulation period. "Stimulated" (S) values are the summed differences between the percent fractional release during stimulation and the appropriate SP value.

The effects of drugs can be reported as S_2/S_1 ratios. To normalize the data, drug effects can be estimated by first calculating S_2/S_1 values for control and drug-treated slices and then expressing the S_2/S_1 value for the drug-treated slices as a percentage of the S_2/S_1 value for the control slices for each experiment. Each condition should be tested in slices from the same animal.

MODIFICATIONS OF THE METHOD

Release of other neurotransmitters from brain tissue in vitro

Several authors (Harms et al. 1979; de Belleroche and Gardiner 1982; James and Cubeddu 1984; Raiteri

et al. 1984; Zahniser et al. 1986; Smith et al. 1984, 1994) studied the release of neurotransmitters from brain tissue *in vitro*.

Raiteri et al. (1974) described a simple apparatus for studying the release of neurotransmitters from synaptosomes.

De Boer et al. (1988) determined the release of noradrenaline and serotonin in synaptosomes from rat cerebral cortex.

Saijoh et al. (1985) studied the influence of hypoxia on release and uptake of the neurotransmitters dopamine and acetylcholine in guinea pig striatal slices.

PROCEDURE

[^{3}H]Norepinephrine release from cortical slices

Cortical slices (0.4 mm) from male Wistar rats are preincubated in Krebs buffer saturated with 95% O_2/5% CO_2, pH 7.4 for 30 min at 35 °C, and then incubated in fresh buffer containing 25 nM [^{3}H]NE (35 Ci/mmol) for 30 min at 35 °C. The slices are then placed in glass superfusion chambers containing platinum electrodes and perfused at 0.75 ml/min. Fractions are collected at 7 min intervals. Slices are electrically stimulated with unipolar pulses (15–30 mA) of 2 ms duration at 5 Hz for 60 s. Two rounds of stimuli are applied, separated by 10 fractions. Test compound is applied at fraction 14 (28 min after the first stimulation).

The fractions collected are counted for tritium in 10 ml of Liqiscint® scintillation fluid and corrected for quench. For measurement of remaining tritium, slices are dissolved overnight in 0.5 ml of Protosol®, buffered with 1 ml of Tris HCl, and counted. Percent fractional release is defined as the ratio of tritium released versus the amount present in the tissue.

[^{3}H]Norepinephrine release from cortical synaptosomes

Cortices from male Wistar rats are homogenized in 9 volumes of 0.32 M sucrose in a Potter-Elvejhem homogenizer and then centrifuged at 1 000 *g* for 10 min at 4 °C. The supernatant is recentrifuged at 17 000 *g* for 20 min, and the pellet is resuspended in 0.32 M sucrose at the original volume.

The freshly prepared synaptosomes are incubated with 50 nM [^{3}H]NE in Krebs buffer containing 10 mM pargyline for 10 min at 37 °C. The ratio of buffer to tissue suspension is 80 : 20. The [^{3}H]NE-loaded synaptosomes are then separated by centrifugation (17 000 *g* for 20 min), washed with buffer containing pargyline, recentrifuged, and then finally resuspended in 0.32 M sucrose at their original volume. The assay mixture consists of 900 ml Krebs buffer containing 10 mM pargyline, 100 ml of [^{3}H]NE-loaded synaptosomes, and 10 ml of vehicle or drug. This mixture is then vortexed and incubated for 5 min at either 37 °C or 0 °C (to define total versus non-specific release). After a 10 min centrifugation (3 000 *g*), the pellets are solubilized in a Triton X-100/ethanol mixture and transferred to scintillation vials, and counted in 10 ml of Liquiscint®.

The net disintegrations for the 37 °C and 0 °C incubations are calculated, and % increase values determined as $[(\text{control} - \text{drug}) / \text{control}] \times 100$.

Several chemical methods to measure acetylcholine (Israël and Lesbats 1982; Damsa et al. 1985; Stadler and Nesselhut 1986), or catecholamines and serotonin (Wagner et al. 1979; Magnusson et al. 1980; Nielsen and Johnston 1982; Wagner et al. 1982) are available.

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F.2.0.6 [^{3}H]Oxotremorine-M binding to muscarinic cholinergic receptors in rat forebrain

PURPOSE AND RATIONALE

The muscarinic receptors are members of the superfamily of G-protein-coupled receptors. They are relatively abundant and mediate the diverse action of acetylcholine in the CNS, as well as throughout non-nervous tissues innervated by the parasympathetic nervous system. Five separate genes (m1–m5) encode muscarinic receptor proteins exhibiting the rhodopsin-like structural motif containing seven transmembrane domains. They show strong sequence homology with each other and with related G-protein-coupled receptors within the transmembrane spanning domains, but each receptor has unique amino acid sequences located at the extracellular amino end and in the third intracellular loop (Caulfield 1993; Jones 1993; McKinney 1993; Wess 1996).

The purpose of this assay is to determine the binding affinity of potential cholinomimetic drugs for muscarinic receptors in brain, using an agonist ligand.

Oxotremorine is a potent centrally and peripherally acting muscarinic cholinergic agonist (Chao et al. 1962; Bebbington et al. 1966), which has been shown to be active in isolated tissue preparations as well as *in vivo* (Rindahl and Jenden 1983). Both central and peripheral effects of oxotremorine are blocked by antimuscarinic drugs such as atropine (Chao et al. 1962; Bebbington et al. 1966). Structural modification of the oxotremorine molecular yields compounds which are full agonists, partial agonists and antagonists at muscarinic receptors (Rindahl and Jenden 1983). Oxotremorine-M (oxo-M), a quaternary nitrogen analog of oxotremorine, is a full agonist for the phosphatidyl-inositol response, while oxotremorine is a partial agonist (Fisher et al. 1984). Both oxotremorine and oxo-M are full agonists for inhibition of adenylate cyclase (Ehlert 1985; Olianas et al. 1983; Brown and Brown 1984). Of the muscarinic agonists, oxotremorine is the most potent inhibitor of [^{3}H]QNB binding, however, the IC_{50} is still only in the micromolar range. The apparent low affinity of agonist competition for [^{3}H]-antagonist binding sites is a common phenomena and is due to the existence of multiple agonist affinity states of the receptor, as described by Birdsall et al. (1978). For this reason, it is desirable to use an agonist ligand to measure the binding affinities of potential agonists.

Molecular methods have disclosed the existence of five muscarinic receptors which are coupled to different second messenger systems. At least four of them are expressed as functional receptor proteins in the neocortex and hippocampus formation (McKinney and Coyle 1991).

PROCEDURE

Reagents

1. 0.5 M Tris buffer, pH 7.4
   66.1 g of Tris HCl
   9.7 g of Tris base
   q.s. to 1 liter with deionized water
2. 0.05 M Tris buffer, pH 7.4
   (10-fold dilution of reagent 1)
3. [Methyl-^{3}H]-oxotremorine acetate (specific activity 83–85 Ci/mmol) is obtained from New England Nuclear.
   For IC_{50} determinations: [^{3}H]Oxotremorine-M is made up to a concentration of 100 nM. Fifty µl of this solution is added to each assay tube (yields a final concentration of 5 nM).
4. Atropine sulfate is obtained from Sigma Chemical Company. A 2 mM stock solution is made up in distilled water. Twenty µl is added to 3 tubes for determination of non-specific binding (yields a final concentration of 40 µM).
5. A 0.5% (w/v) solution of polyethylene-imine is prepared in distilled water. GF/C filters are soaked in this solution for at least three hours at room temperature. This is done to reduce the binding of the ligand to the filter strips.
6. Test compounds: For most assays, a 10 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2×10^{-4} to 2×10^{-7} M.

Tissue preparation

Male Wistar rats are decapitated and their brains rapidly removed. The forebrains (all tissue forward of a vertical cut in front of the hypothalamus) are weighed (400–500 mg each) and homogenized in 10 volumes of 0.05 M Tris buffer, pH 7.4 (reagent 2) using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate is centrifuged at 1 000 *g* for 10 min. The supernatant is then centrifuged at 50 000 *g* for 60 min. The supernatant from this centrifugation is discarded and the pellet resuspended in the original volume of 0.05 M Tris buffer, pH 7.4, on the Polytron to 100 mg/ml (wet weight). Specific binding is roughly 1% of total added and 50% of total bound.

Binding assay

50 µl 0.5 M Tris buffer, pH 7.4 (reagent 1)
380 µl H_2O
20 µl drug or 2 mM atropine
50 µl ^{3}H-oxotremorine-M (reagent 3)
500 µl tissue

Tubes are vortexed and incubated at 30 °C for 45 min (2). Bound [^{3}H]-oxotremorine-M is captured by filtration under reduced pressure. The incubation mixture is diluted with approximately 4 ml ice-cold 0.05 M Tris buffer, pH 7.4 (reagent 2), then exposed to vacuum, and tubes washed once more with approximately 5 ml of reagent 2. The filters (GFC in 0.5% polyethylene-imine for more than 3 h, reagent 5) are then counted in 10 ml Liquiscint scintillation fluid.

EVALUATION

Specific binding is the difference between total bound (in presence of vehicle) and that bound in the presence of 40 µM atropine. Percent inhibition of specific [^{3}H]-oxotremorine-M is calculated for each concentration of test drug and IC_{50} values determined by computer-derived log probit analysis. The percent inhibition at each drug concentration is the mean of duplicate or triplicate determinations. Some day-to day variability is present in this assay, and IC_{50} values should be confirmed by repeat analysis.

MODIFICATION OF THE METHOD

[^{3}H]Pirenzepine has been used to identify muscarinic receptor subtypes in the brain (Watson et al. 1983a,b).

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F.2.0.7 [^{3}H]-N-Methylscopolamine binding in the presence and absence of Gpp(NH)p

PURPOSE AND RATIONALE

G-protein-linked muscarinic receptors are converted by guanine nucleotides from a high-affinity binding state to a low-affinity binding state for muscarinic agonists (Gilman 1986), while the binding of muscarinic antagonists to the receptor is not affected. The effects of guanine nucleotides on muscarinic agonist affinity are brain region- and temperature-dependent (Aron-stram and Narayanan 1988). Therefore, incubation of cerebellar membranes with 50 μM 5'-guanylylimidophosphate (Gpp(NH)p), the non-hydrolyzable analog of GTP, causes a shift to the right (decreased affinity) of the muscarinic agonist inhibition curves when ^{3}H-NMS is used as the ligand.

The assay differentiates the interaction of muscarinic agonists and muscarinic antagonists with ^{3}H-N-methylscopolamine (^{3}H-NMS)-labeled receptors in cerebellar tissue based on the selective effect of guanine nucleotides on the affinity of muscarinic agonists for the receptor.

PROCEDURE

The procedure is based on ^{3}H-NMS rat brain binding assay described by Aronstram and Narayanan (1988).

Reagents

1. 0.5 M Tris-HCl buffer, pH 7.4
2. 0.05 M Tris-HCl buffer, pH 7.4
3. 0.05 M Tris-HCl buffer, pH 7.4 + 2 mM $MgCl_2$
4. 0.05 M Tris-HCl buffer, pH 7.4 + 2 mM $MgCl_2$ + 100 μM phenylmethylsulfonyl fluoride
5. Atropine sulfate is made up to 1 mM in distilled water and 20 μl is added to a 2 ml reaction mixture. This yields a final concentration of 10 μM. Atropine is used for nonspecific binding.
6. 5'-Guanylylimidodiphosphate (Gpp(NH)p) is made up to 2 mM in distilled water. The final concentration in the reaction mixture is 50 μM.

7. ^{3}H-N-Methylscopolamine (NMS) is obtained from Amersham and diluted to 4 nM in distilled water. The final concentration in the reaction mixture is 0.1 nM.
8. Test compounds
   For most assays, a 10 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-4} to 10^{-7} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are decapitated and their brains rapidly removed. The cerebella are dissected, weighed and homogenized in 10 volumes of 0.05 M Tris-HCl buffer, pH 7.4 + 2 mM $MgCl_2$ + 100 µM phenylmethylsulfonyl fluoride (buffer 4), using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate is centrifuged at 20 000 *g* for 20 min. The pellet is resuspended in 10 volumes of 0.05 M Tris-HCl buffer + 2 mM $MgCl_2$ (buffer 3).

Binding Assay

1 000 µl	0.05 M Tris buffer + 2 mM $MgCl_2$
780 µl	H_2O
20 µl	vehicle or 1 mM atropine or appropriate drug concentration
50 µl	H_2O or Gpp(NH)p
50 µl	[^{3}H]NMS
100 µl	tissue suspension

Tubes are incubated at 20 °C for 90 min. Bound [^{3}H]NMS is captured by vacuum filtration. The filters are washed three times with 5 ml aliquots of 0.05 M Tris buffer, pH 7.4. Filters are counted in 10 ml Liquiscint scintillation fluid.

EVALUATION

Specific binding of [^{3}H]NMS is the difference between total bound (in the presence of vehicle) and that bound in the presence of 1 mM atropine. Percent inhibition of specific [^{3}H]NMS binding is calculated for each concentration of test drug and IC_{50} values are determined by computer-derived log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

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F.2.0.8 Stimulation of phosphatidylinositol turnover in rat brain slices

PURPOSE AND RATIONALE

The purpose of this assay is to determine the ability of test compounds to stimulate the turnover of phosphatidylinositol (PI) in brain tissue. This assay can be used to determine agonist activity at a number of CNS receptors known to be linked to the PI response. A major interest is the evaluation of muscarinic cholinergic receptors.

Receptor-activated hydrolysis of inositol phospholipids is now recognized as an important second messenger system for muscarinic, alpha-adrenergic, histaminergic, serotonergic, excitatory amino acid and various neuropeptide receptors (Berridge and Irvine 1984; Nahorski et al. 1986; Fisher and Agranoff 1987). Furthermore, the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) yields at least two important biologically-active intermediates (Hirasawa and Nishizuka 1985; Berridge 1987). These include 1,4,5-inositol trisphosphate (IP_3), which acts to mobilize Ca^{2+} from the endoplasmic reticulum and diacylglycerol, which activates protein kinase C (PKC). These responses are associated with many cellular responses such as stimulus-secretion coupling, stimulus-contraction coupling and cell proliferation. The exact mechanism for receptor-mediated turnover of cell membrane PI is not well understood, but it seems to involve coupling through a G-protein (Cockcroft and Gomperts 1985) and requires extracellular Ca^{2+} to activate phospholipase C (Fisher et al. 1989).

Although the muscarinic receptor-PI link has been known for some time (Hokin and Hokin 1955), recent advances in knowledge of receptor mechanisms and increased interest in the muscarinic receptor have stimulated considerable research in this area. Even though muscarinic agonists can be shown to be weak partial agonists or full agonists for this response (Fisher et al. 1983) and there are brain regional differences in sensitivity (Fisher and Bartus 1985); attempts to show receptor subtype selectivity for stimulation of PI turnover in either heart (Brown et al. 1985) or brain tissue (Fisher and Bartus 1985) have been disappointing. However, recent experiments using cells transfected with genomic clones for the various muscarinic receptors have had more success (Shapiro et al. 1988; Conklin et al. 1988). These studies show that the m_1, m_3 and the m_5 receptor subtypes are linked to PI turnover.

Stimulation of PI turnover by agents such as veratridine, batrachotoxin and ouabain (Gusovsky et al. 1986) show that there are also non-receptor mechanisms that can cause the stimulation of PI turnover.

PROCEDURE

Equipment and materials

1. McIlwain tissue slicer
2. Disposable columns (Kontes, 200 mm)
3. Column rack (Kontes)
4. Disposable screw-cap tubes (Pyrex, 16 × 100 mm)
5. Disposable culture tubes (Fisher, 16 × 125 mm)

Reagents

1. Modified Krebs bicarbonate buffer

	g/l	mM
NaCl	8.30	142.0
KCl	0.42	5.6
$CaCl_2$	0.24	2.2
$NaHCO_3$	0.30	3.6
$MgCl_2 \cdot 6H_2O$	0.20	1.0
HEPES	7.15	30.0
Adjust pH to 7.4 with NaOH		
d-Glucose[a]	1.01	5.6

[a] Glucose added just before incubation.

2. Concentrated Krebs buffer + LiCl (9-fold concentrated ions, 10-fold concentrated Li^+), stock solution contains no glucose or Ca^{2+}.

	g/100 ml
NaCl	6.88 (amount adjusted to correct for Li^+)
KCl	0.38
$NaHCO_3$	0.27
$MgCl_2 \cdot 6H_2O$	0.18
HEPES	6.44
LiCl	0.42

   $CaCl_2$ (11 mg) and d-glucose (45 mg) are added to 5 ml of concentrated buffer before incubation.
3. Dowex AG-1-X8 (100–200 mesh) formate form (Biorad)
4. [^{3}H]Inositol (spec. act. 15 Ci/mmol) is obtained from American Radiolabeled Chemicals, Inc.
5. Myo-inositol (M.Wt. 180.2) is obtained from Sigma Chemical Co. A 5 mM solution is made (0.9 g/l).
6. 1 M Ammonium formate/0.1 M formic acid. Ammonium formate is obtained from Sigma Chemical Co.: 3.85 ml 99% formic acid + 63.1 g ammonium formate to 1 liter in H_2O.
7. $CHCl_3$/methanol (1 : 2, v/v).

Tissue preparation

Male Wistar rats, approximately 6/assay.

1. Remove surface blood vessels by rolling brain on filter paper.
2. Remove cerebral cortex and gently scrape myelin layer off.
3. Prepare 350 × 350 micron tissue slices with the McIlwain tissue chopper and place slices in buffer at 37 °C.
   Incubate for 10 min.
4. Disperse slices by aspirating into 1-ml pipet (cut off tip).
5. Allow slices to settle, aspirate supernatant, add buffer and repeat until the supernatant is clear.

Assay: Prelabeled method

1. Add to screw-cap tubes:
   50 µl tissue slices
   350 µl [^{3}H]inositol
2. Incubate for 90 min at 37 °C under O_2
3. Add:
   50 µl drug
   H_2O to a final volume of 500 µl
4. Incubate for 30 min at 37 °C under O_2.
5. Stop the reaction by the addition of 1.5 ml of $CHCl_3$: MeOH. Place in ice bath.

Assay: Continuous labeling method

1. Add to screw-cap tubes:
   50 µl tissue slices
   350 µl [^{3}H]inositol
   50 µl drug
   H_2O to a final volume of 500 µl
2. Incubate for 120 min. at 37 °C under O_2.
3. Stop the reaction by the addition of 1.5 ml of $CHCl_3$: MeOH. Place in ice bath.

For antagonist inhibition studies, the antagonist is usually preincubated with the slices before the agonist is added.

Extraction of total [^{3}H]inositol phosphates

1. Add 1 ml of $CHCl_3$ and 0.5 ml of H_2O to each tube. Cap and vortex.
2. Centrifuge at 3 000 rpm for 10 min.
3. Aspirate the aqueous phase and place in culture tubes.
4. Add 1.7 ml of H_2O and heat in water bath at 55 °C for 20 min.

5. Place samples in cold room overnight.
6. Add 0.5 ml of Dowex 50% slurry to each tube. Vortex 4 times.
7. Centrifuge at 3 000 rpm for 10 min.
8. Aspirate.
9. Add 2.5 ml of 5 mM myo-inositol, let the resin settle and aspirate. Repeat 5 times.
10. Centrifuge at 3 000 rpm for 10 min.
11. Add 1 ml of ammonium formate/formic acid, pH 4.8.
12. Put 0.7 ml of supernatant into scintillation vial, add 10 ml cocktail and count.

EVALUATION

The stimulation phosphatidylinositol turnover for each test compound is calculated as percent increase in total [^{3}H] inositol phosphates relative to the basal turnover rate of non-treated control brain slices. The EC_{50} values for agonists are determined by log-probit analysis of these data. IC_{50} values for antagonists are determined by log-probit analysis of the percent inhibition of stimulation by a full agonist.

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F.2.0.9 [^{3}H]N-Methylcarbamylcholine binding to nicotinic cholinergic receptors in rat frontal cortex

PURPOSE AND RATIONALE

The purpose of this assay is to determine the binding affinity of potential nicotinic cholinergic agonists in brain, using an agonist ligand.

Nicotinic cholinergic receptors are classified as ligand-gated ion channels, and are found in skeletal muscle, autonomic ganglia and brain tissue. Nicotine itself has a variety of behavioral effects. Due to its rapid desensitization of the receptor, both stimulatory and depressant effects may result. Also, many of nicotine's effects are thought to be associated with release of neurotransmitter substances (Balfour 1982). Nicotine functions as a nicotinic cholinergic receptor agonist in the CNS and is thought to play a role in learning and memory (Clarke 1987). Reductions in nicotinic binding sites were found in post-mortem tissues from Alzheimer's patients by four separate groups of investigators (Whitehouse et al. 1988; Nordberg and Winblack 1986; Araujo

et al. 1988; Shimohama et al. 1986). Cognitive improvement in SDAT EXPLAIN? patients thirty minutes after a nicotine infusion was reported (Sunderland et al. 1988). Therefore nicotinic agonists may prove beneficial, however, clinical data are still quite limited. N-Methylcarbamylcholine (NMCC) is a nicotinic agonist which binds specifically and with high affinity to central nicotinic recep-tors and, like nicotine, causes an increase of acetylcholine release from certain cholinergic nerve terminals (Araujo et al. 1988; Lapchak et al. 1989). In addition, chronic nicotine treatment increases [^{3}H] NMCC binding sites in several rat brain regions, as it does with [^{3}H]nicotine and [^{3}H]ACh labeled sites (Lapchak et al. 1989). This is due to the loss of presynaptic nicotinic autoreceptor function (Lapchak et al. 1989). Pharmacological results reveal that along with its specificity and high affinity, [^{3}H]NMCC is selectively displaced by agonists (Araujo et al. 1988; Lapchak et al. 1989), making it a desirable ligand to screen for potential agonistic compounds.

Nicotinic acetylcholine receptors are a family of ligand gated ion channels that are classified on the basis of their activation by nicotine, although acetylcholine is the endogenous ligand. These conductance channels for Ca^{2+}, K^+ and Na^+ are pentameric in structure. They are members of a supergene family that also includes glycine, $GABA_A$, and 5-HT3 receptors. α, β, γ, and δ subunits constitute a pentameric neuronal receptor resulting in various receptor subtypes (Sargent 1993; Alkondon and Albuquerque 1993). Nine α (α1–α9) and three β (β2–β4) subunits have been cloned from mammalian and avian sources, each of which has a structural motif of four transmembrane spanning domains M1–M4 of which M2 lines the channel (Gotti et al. 1997).

PROCEDURE

Reagents

1. 0.5 M Tris buffer, pH 7.7
   a) 57.2 g Tris HCl
      16.2 g Tris Base
      q.s. to 1 liter with distilled water
   b) Make a 1 : 10 dilution in distilled H_20 (0.05 M Tris buffer, pH 7.7 at 25 °C)
2. Tris buffer containing physiological ions
   a) Stock buffer

NaCl	7.014 g
KCl	0.372 g
$CaCl_2$	0.222 g
$MgCl_2$	0.204 g q.s. to 100 ml with 0.5 M Tris buffer

   b) Dilute 1 : 10 in distilled H_20
      This yields 0.05 M Tris HCl, pH 7.7, containing NaCl (120 mM), KCl (5 mM), $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM).
3. Methylcarbamoylcholine iodide, [N-Methyl-^{3}H]- is obtained from New England Nuclear
   For IC_{50} determinations [^{3}H]-NMCC is made up to a concentration of 100 nM in distilled H_2O and 50 µl added to each tube (yields a final concentration of 5 nM in the 1 ml assay).
4. (–)Nicotine ditartrate is obtained from Research Biochemicals Incorporated.
   A stock solution of (–)nicotine ditartrate is made up to a concentration of 0.5 mM in distilled H_2O. Twenty µl of stock is added to 3 tubes for the determination of nonspecific binding (yields a final concentration of 10 µM in the assay).
5. Test compounds. For most assays in 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2×10^{-5} to 2×10^{-8} M. Seven concentrations are used for each assay and higher or lower concentrations may be used depending on the potency of the drug.
6. A 0.5% (w/v) solution of polyethyleneimine is prepared in distilled H_2O. GF/B filters are soaked in this solution for at least four hours at 4 °C. This is done to reduce binding of the ligand to the filter strips.

Tissue preparation

Male Wistar rats are decapitated, their frontal cortices removed, weighed and homogenized in 40 volumes of ice-cold 0.05 M Tris buffer, pH 7.7 (1 b). The homogenate is centrifuged at 48 000 *g* for 10 min. The pellet is rehomogenized in fresh buffer and recentrifuged at 48 000 *g* for 10 min two more times. The final pellet is resuspended in the original volume of buffer, but with physiological salts (2 b). This yields a final tissue concentration of 20 mg/ml in the assay.

Assay

130 µl	0.05 M Tris pH 7.7-physiological salts (2 b).
20 µl	Vehicle (for total binding) or 0.5 mM (–)nicotine ditartrate (for nonspecific binding) or appropriate drug concentration.
50 µl	N-[^{3}H]Methylcarbamylcholine stock solution
800 µl	tissue.

The tubes are incubated at 0 °C for 60 min. The assay is stopped by rapid filtration through Whatman GF/B filters which are then washed 4 times with 3 ml of ice-cold 0.05 M Tris buffer, pH 7.7. The filters are then counted in 10 ml of Liquiscint scintillation cocktail.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of the 10 µM

(–)nicotine ditartrate. Specific binding is about 1% of the total added ligand and 60–70% of the total bound ligand. IC_{50} calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

MODIFICATION OF THE METHOD

Pabreza et al. (1991) recommended [^{3}H]cytisine as a useful ligand for studying neuronal nicotinic receptors because of its high affinity and low nonspecific binding.

Badio and Daly (1994) determined [^{3}H]nicotine receptor binding in rat cerebral cortex membrane preparations. The authors concluded that the analgesic activity of epibatidine, an alkaloid originally characterized from frog skin, is due to its activity as nicotinic agonist.

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F.2.0.10 Cultured neurons/astroglial cells

PURPOSE AND RATIONALE

Cultured brain cells can be used for many purposes such as investigation of synthesis and secretion of nerve growth factor or for testing neuroprotective drugs (Peruche and Krieglstein 1991). Nerve growth factor is required for the development and maintenance of peripheral and sensory neurons (Thoenen and Barde 1980). Nerve growth factor prevents neuronal death after brain injury (Hefti 1986; Williams et al. 1986; Kromer 1987), especially in basal forebrain nuclei involved in memory processes. Drug induced increase in nerve growth factor secretion may be beneficial in primary degenerative dementia.

Nerve growth factor belongs to the family of neurotrophins which includes besides of the nerve growth factor, the neurotrophin 3 (NT3), neurotrophin 4/5 (NT 4/5) and brain-derived neurotrophic factor (BDNF). Two types of neurotrophin transmembrane receptors are known: (1) a receptor termed p75, which is common to all neurotrophins and (2) a family of neurotrophin receptor tyrosine kinases trkA, trkB and trkC (Saragovi and Gehring 2000).

PROCEDURE

Whole brains of 8-day-old mice (ICR) are dissected out and cut into small pieces. The pieces are washed

with calcium- and magnesium-free phosphate-buffered saline, treated with 0.25% trypsin at 37 °C for 30 min, and triturated with a Pasteur pipette. The excess trypsin is removed by centrifugation at 200 *g* for 5 min. The cells or cell clumps from one brain are cultured in a culture bottle with Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS), 50 milliunits/ml penicillin, and 50 mg/ml of streptomycin at 37 °C in a humid atmosphere of 5% CO_2 for 1–2 weeks with medium changes every 3 days. After confluence is reached, the cells in each bottle are dissociated by trypsin treatment and recultured in new bottles. This procedure is repeated 3 times. The culture becomes composed of morphologically uniform cells.

Preparation of quiescent cells is performed by inoculating into 96-well plates and culture in FCS-containing DMEM until confluence is reached. Then, the cells are cultured for an additional week in FCS-free DMEM containing 0.5% BSA, with medium changes every 3 days. Because the cells never proliferate in FCS-free medium, most of the cells are arrested in the quiescent phase. Then, the medium is changed to DMEM containing 0.5% BSA with or without drugs, and the cells are cultured for 24 h.

Nerve growth factor (NGF) content in the culture medium is determined by a two-site enzyme immunoassay (Furukawa et al. 1983; Lärkfors and Ebendal 1987). Mouse β NGF isolated from male mouse submaxillary glands is purified by CM-Sephadex C-50 chromatography. Antiserum to this mouse β NGF is produced in New Zealand White rabbits by repeated subcutaneous injections of an emulsion in complete Freund's adjuvant over 18 months. Immunoglobin G is prepared from Anti-mouse β NGF antiserum by Sepharose chromatography. Antibody IgG is incubated with pepsin and chromatographed. Fab' fragments are coupled to β-D-galactosidase. IgG-coated solid phase is prepared in polystyrene tubes. The IgG-coated polystyrene tubes are incubated with 0.25 ml buffer containing various amounts of NGF with gentle shaking. After incubation for 18–24 h at 4 °C, each tube is washed twice with 1 ml of buffer and 0.13 milliunits of the Fab'-β-D-galactosidase complex in 0.25 ml buffer is added. After incubation for 18–24 h at 4 °C with gentle shaking each tube is washed as described above and β-D-galactosidase activity bound to the tube is assayed. The enzyme reaction is started by addition of 60 mM 4-methylumbelliferyl-β-D-galactoside and 0.1% Triton X-100 in 0.25 ml buffer. After 1-h incubation at room temperature, the enzyme reaction is stopped by the addition of 1.25 ml 0.1 M glycine-NaOH buffer (pH 10.3). The amounts of 4-methylumbelliferone formed are measured by fluorometry (Excitation wavelength 360 nm, emission wavelength 450 nm).

EVALUATION

Time-response curves of release of NGF into the medium are established after addition of drug and compared with controls. Dose-response curves can be prepared after addition of various amounts of test drug.

MODIFICATIONS OF THE METHOD

Cultured neurons from chick embryo hemispheres were used for testing cerebroprotective drug effects *in vitro* and for testing antihypoxic drug effects by Krieglstein et al. (1988), Peruche et al. (1990), Oberpichler-Schwenk and Krieglstein (1994).

Semkowa et al. (1996) found that clenbuterol protects mouse cerebral cortex and rat hippocampus from ischemic damage and attenuates glutamate neurotoxicity in cultured hippocampal neurons by induction of nerve growth factor.

Prehn et al. (1993, 1995) tested the prevention of glutamate neurotoxicity in neocortical cultures from rats. Mixed neuronal/glial primary cultures were derived from the cerebral cortices of neonatal Fischer 344 rats. Excitotoxic injury was induced after 14 days by L-glutamate following a procedure described by Choi et al. (1988), Koh and Choi (1988).

Kinoshita et al. (1991) used primary cultured neurons from 17-day-old rat fetuses.

Qi et al. (1997), Horton et al. (2001) described a novel catecholaminergic CAD CNS neuronal cell line in which neurotrophin-3 mediates the autocrine survival.

Shinpo et al. (1999) used cultured mesencephalic neurons from embryonic Sprague Dawley rats to study the protective effects of the TNF-ceramide pathway against glutamate neurotoxicity.

Matsumoto et al. (1990) described a method for quantifying the effects of neurotrophic factors on the number of surviving neurons and the total length of neurites in primary cultures from cerebral cortex and hippocampus of the brains from two-week old rats by using digital image processing techniques. Binary images of neuronal neurites were extracted from gray images of cultured neurons stained with Coomassie brilliant blue.

White et al. (1995) measured calcium transients in mouse cerebellar granule cells with the Ca^{2+}-sensitive probe indo-1/AM.

Beresini et al. (1997) developed two types of high throughput assays to identify small molecules that interact with neurotrophin receptors. The first, the receptor binding assay, is a competitive binding assay that uses a recombinant receptor fusion protein and biotinylated neurotrophin. This assay detects compounds that inhibit neurotrophin binding to the receptor; these compounds may be either agonistic or antagonistic. The second assay, the kinase receptor activation ELISA,

detects receptor autophosphorylation in response to sample- or neurotrophin-stimulation of receptor transfected cells. Receptor autophosphorylation is evaluated by analyzing lysates of the stimulated cells in a receptor-specific ELISA for phosphotyrosine residues. This assay is bioactivity based, and consequently, has the power of detecting as well as distinguishing receptor agonists and antagonists.

Höglinger et al. (1998) used free-floating roller tube cultures prepared from embryonic-day-14 rat ventral mesencephalon to study the influence of brain-derived neurotrophic factor treatment on dopamine neuron survival and function.

Nerve growth factor is crucial for survival of nociceptive neurons during development. Shu and Mendell (1999) investigated the acute effects of NGF on capsaicin responses of small-diameter dorsal root ganglion cells in culture.

For further studies with brain cell cultures see E.3.1.16.

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F.2.0.11 Inhibition of respiratory burst in microglial cells/macrophages

PURPOSE AND RATIONALE

Activated phagocytes can produce large amounts of oxygen intermediates that result from a process during which NADPH oxidase reduces O_2 to the superoxide anion (O_2^-), which subsequently dismutates together with H^+ to H_2O_2 and O_2. H_2O_2 is then reduced to hypochlorous acid by myeloperoxidase (Bellavite 1988). The cascade of metabolic steps is known as respiratory bursts. The intracellular formation of reactive oxygen intermediates can be determined by measuring the oxidation of the membrane permeable and non-fluorescent dihydrorhodamine 123 to the cationic and intracellularly trapped, green fluorescent rhodamine 123 in single viable cells. Microglial/brain macrophage-mediated damage in the central nervous system is accompanied by an increased production of free radicals, which also seems important in primary degenerative dementia (Alzheimer's disease) (Banati et al. (1993). Inhibition of this process by drugs may indicate therapeutic value in Alzheimer's disease.

PROCEDURE

Cell culture

Cultures of new-born rat brain are prepared as described by Guilian and Baker (1986); Frei et al. (1987). Isolated cerebral cortices from new-born albino rats are stripped of the meninges, minced in culture medium and dissociated by trituration for 2 h in 0.25% trypsin solution. Cells are plated in 75 cm^2 plastic culture flasks containing 10 ml medium with 10% fetal bovine serum at a density of 85 000 cells/ml. After 7 days, confluent cultures are vigorously agitated on a rotary shaker at 37 °C for 15 h. Glial fibrillary acid protein positive astroglia remain adherent to the flasks. The resulting cell suspension, rich in ameboid microglia and oligodendroglia, is placed in plastic flasks and allowed to adhere at 37 °C. After a 1–3 h adhering interval, loosely adhering and suspended cells (most of which are oligodendroglia) are removed by gently shaking the flasks at room temperature. The strongly adherent microglia cells are then released by vigorous shaking in medium with 0.2% trypsin. Once the majority of microglia is suspended, fetal bovine serum is added (15% final volume), and the cell suspension added to new flasks. After a second 1–3 h interval to allow adhesion, the medium is removed, and adhering microglia are suspended using trypsin. Final preparation show a nearly homogeneous population of non-specific esterase positive cells.

Peritoneal macrophages are obtained from 12-week-old male Wistar rats.

For flow cytometric measurement, cells are suspended ($3–4 \times 10^6$ cells/ml) in Hank's buffered saline (HBS; Sigma Chemie, Deisenhofen, Germany) supplemented with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; 5 mM, pH 7.35; Serva Feinbiochemica, Heidelberg, Germany) (HBS-HEPES) and stored at 4 °C for a maximum of 2 h.

Flow cytometric measurement of respiratory burst

Dihydrorhodamine 123 (DHR) is obtained from Molecular Probes (Eugene, OR, USA) and dissolved to obtain a 1 mM stock solution in N,N-dimethylformamide (DMF; Merck, Darmstadt, Germany).

The cellular suspensions of peritoneal macrophages and microglial cells (10 µl) are each further diluted with 1 ml HBS-HEPES and stained for 5 min at 37 °C with 10 µl of a 100 µM DHR solution in HBS (1 mM stock solution in DMF). The DHR-loaded cells are incubated with the test drug at various concentrations for 15, 25, 35, 45, and 60 min with and without Con A (100 µg/ml; Sigma Chemie) stimulation. The DNA of dead cells is counter stained with 10 µl of 3 mM propidium iodide (Serva Feinbiochemica) solution in HBS 3 min before the flow cytometric measurement. To exclude effects from a possible release of endogenous adenosine, control experiments with incubation medium containing adenosine deaminase (200 U/mg, 5 µg/ml Sigma Chemie) are performed.

The forward scatter, side scatter and two fluorescences of at least 10 000 cells/sample are measured simultaneously on an FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Rhodamine 123 green fluorescence (515–545 nm) and propidium iodide red fluorescence (>650 nm) are measured with the light from an argon laser of 488-nm excitation wavelength.

EVALUATION

The differences in respiratory burst activities caused by Con A stimulation and treatment with test drug are

tested for significance by unpaired *t*-test. The *t*-test is performed for each time point including the data of at least four independent experiments. Each single fluorescence value of each experiment is based on the measurement of at least 10 000 cells. Before each experiment the flow cytometer is calibrated with standardized yellow-green fluorescent microspheres of 4.3-µm diameter (Polysciences, St. Goar, Germany), thus ensuring the compatibility of the fluorescence values from different experimental series.

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F.3 *In vivo* methods

F.3.1 Inhibitory (passive) avoidance

One of the most common animal tests in memory research is the inhibition to imitate activities or learned habits. The term "passive avoidance" is usually employed to describe experiments in which the animal learns to avoid a noxious event by suppressing a particular behavior. Netto and Izquierdo (1985) have discussed this term in a brief paper. The term "inhibitory avoidance" is used more often in later publications.

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F.3.1.1 Step-down

PURPOSE AND RATIONALE

An animal (mouse or rat) in an open field spends most of the time close to the walls and in the corners. When placed on an elevated platform in the center of a rectangular compartment, it steps down almost immediately to the floor to explore the enclosure and to approach the wall. The technique is employed in different modifications, which are described by Jarvik and Essmann (1960), Hudspeht et al. (1964), Chorover and Schiller (1965), Kubanis and Zornetzer (1981), Zornetzer et al. (1982), Abdel-Hafez et al. 1998).

PROCEDURE

Mice or rats of either sex are used. A rectangular box (50×50 cm) with electrifiable grid floor and 35 cm fits over the block. The grid floor is connected to a shock device which delivers scrambled foot shocks. The actual experiments can be performed in different ways. A typical paradigm consists of three phases: (1.) Familiarization: The animal is placed on the platform, released after raising the cylinder, and the latency to descend is measured. After 10 s of exploration, it is returned to the home cage. (2.) Learning: Immediately after the animal has descended from the platform an unavoidable footshock is applied (Footshock: 50 Hz; 1.5 mA; 1 s) and the animal is returned to the home cage, (3.) Retention Test: 24 h after the learning trial the animal is again placed on the platform and the step-down latency is measured. The test is finished when the animal steps down or remains on the platform (cut-off time: 60 s).

EVALUATION

The time of descent during the learning phase and the time during the retention test is measured. A prolongation of the step-down latency is defined as learning.

MODIFICATIONS OF THE TEST

This test procedure is employed in different modifications. One of the most common modifications is to induce amnesia in animals. There are different methods to do so including (i) electroconvulsive shock, (ii) scopolamine, (iii) alcohol, (iv) CO_2.

CRITICAL ASSESSMENT OF THE METHOD

The variability of this method is relative high, therefore, it is necessary to test large groups of animals (minimum 10 animals per group). There are some critical parts in the experimental procedure: (i) Placing the animal on the platform, since the tendency of the ani-

mal to escape the contact with the human hand may shorten the step-down latencies. (ii) Another important point is the timing of the electric shock. It must not be applied at the first contact of the animal with the floor, since the light touch with the forelimbs does not cause the required shock intensity. The duration and intensity of the shock should be constant. (iii) It is also necessary to keep the room sound-proof, and this can be done by using a white noise generator (60–70 dB). Due to all these critical variables the results of different authors are difficult to compare.

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F.3.1.2 Step-through

PURPOSE AND RATIONALE

This test uses normal behavior of mice and rats. These animals avoid bright light and prefer dim illumination. When placed into a brightly illuminated space connected to a dark enclosure, they rapidly enter the dark compartment and remain there. The standard technique was developed for mice by Jarvik and Kopp (1967) and modified for rats by King and Glasser (1970). It is widely used in testing the effects of memory active compounds (Fekete and de Wied 1982; Hock and McGaugh 1985; Hock et al. 1989; Hock 1994).

PROCEDURE

Mice and rats of either sex are used. The test apparatus consists of a small chamber connected to a larger dark chamber via a guillotine door. The small chamber is illuminated with a 7 W/12 V bulb. The test animals are given an acquisition trial followed by a retention trial 24 h later. In the acquisition trial the animal is placed in the illuminated compartment at a maximal distance from the guillotine door, and the latency to enter the dark compartment is measured. Animals that do not step through the door within a cut-off time: 90 s (mice) or 180 s (rats) are not used. Immediately after the animal enters the dark compartment, the door is shut automatically and an unavoidable footshock (Footshock: 1 mA; 1 s – mice; 1.5 mA; 2 s – rat) is delivered. The animal is then quickly removed (within 10 s) from the apparatus and put back into its home cage. The test procedure is repeated with or without drug. The cut-off time on day 2 is 300 s (mice) or 600 s (rats), respectively.

EVALUATION

The time to step-through during the learning phase is measured and the time during the retention test is measured. In this test a prolongation of the step-through latencies is specific to the experimental situation. An increase of the step-through latency is defined as learning.

CRITICAL ASSESSMENT OF THE METHOD

Same as in step-down method

MODIFICATIONS OF THE TEST

This test procedure is employed in different modifications. To test drugs usually several test-groups can be tested. (i) CXC-group: vehicle – without amnesia – vehicle; (ii) DXD-group: drug – without amnesia – drug; (iii) CAC-group: vehicle – amnesia – vehicle; (iv) DAD-group: drug – amnesia – drug; (v) CAD-group: vehicle – amnesia – drug; (vi) DAC-group: drug – amnesia – vehicle. (The first figure means treatment on day 1; the third figure means treatment on day 2). There are different methods to induce amnesia: (i) electroconvulsive shock, (ii) scopolamine, (iii) al-cohol, (iv) CO_2 etc.

Step-through experiments were performed after unilateral ibotenic acid lesions in the right nucleus basalis magnacellularis as a rat model of Alzheimer disease by Fine et al. (1985).

Wan et al. (1990) recorded changes in heart rate and body temperature via a telemetry system during step through passive avoidance behavior in rats.

Picciotto et al. (1995) used gene targeting to mutate β2, the most widely expressed neuronal nicotinic acetylcholine receptor in the central nervous system for pharmacological and behavioural studies. Retention of the inhibitory avoidance response was better in mutant mice than in their non-mutant siblings.

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F.3.1.3 Two compartment test

PURPOSE AND RATIONALE

A rodent in an open field tends to enter any recesses in the walls and to hide there. When placed into a large box, connected through a narrow opening with a small dark compartment, the animal rapidly finds the entrance into the small chamber, enters it and spends most of its time there. The times spent in the large and small compartments are measured. The latency of the first entrance into the dark chamber and the number of crossings from one compartment into the other can be used as auxiliary criteria. The technique described was developed by Kurtz and Pearl (1960) and modified by Bures and Buresova (1963).

PROCEDURE

Mice and rats of either sex and a rectangular box with a 50×50 cm grid floor and 35 cm high walls are used. In the centre of one wall is a 6×6 cm opening connecting the large compartment to a small 15×15 cm box with dark walls, electrifiable grid floor and removable ceiling. The connection between the two compartments can be closed with a transparent sliding door. Illumination is provided with a 100 W bulb placed 150 cm above the centre of the large compartment.

EVALUATION

The times the animal spends in the large and the small compartment are measured.

CRITICAL ASSESSMENT OF THE METHOD

Same as in step-down method.

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F.3.1.4 Up-hill avoidance

PURPOSE AND RATIONALE

Many animal species exhibit a negative geotaxis, i.e. the tendency to orient and move towards the top when placed on a slanted surface. When placed on a tilted platform with head facing down-hill, rats and mice invariably turn around and move rapidly up the incline (Staubli and Huston 1978).

PROCEDURE

Rats of both sex were used and maintained under standard conditions. The experimental apparatus is a 50×50 cm box with 35 cm high opaque plastic walls. The box can be inclined at different angles. The floor consists of 10 mm diameter stainless steel grid bars placed 13 mm apart. To deliver the tail-shock, a tail-electrode is constructed, consisting of a wire clip connected to a constant current shock source. The animal is first fitted with the tail-electrode and then placed onto the grid with its nose facing down. During baseline-trials the animal's latency to make a 180° turn and ini-

tiate the first climbing response is measured. Thereafter the animal is returned to its home cage. During the experimental trials the latencies are measured and additionally a tail-shock (1.5 or 2 mA) was administered contingent on the first climbing response after the 180° turn. Immediately after the shock the animal is placed in its home cage. Retest is performed 24 h later.

EVALUATION
The latencies are measured.

CRITICAL ASSESSMENT OF THE METHOD
The up-hill avoidance technique promises to provide a useful addition to the existing arsenal of inhibitory (passive) avoidance methods. Its most obvious advantage is that it can be administered to animals debilitated in sensory-motor coordination by pharmacological or surgical treatments that would preclude use of other inhibitory (passive) avoidance tasks.

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F.3.1.5 Trial-to-criteria inhibitory avoidance

PURPOSE AND RATIONALE
As animals experience different sensitivity to the footshock punishment applied in the dark area, immediately after the first trial the animal is returned to the lighted area to evaluate if the task has been acquired. A criteria is established to determine the learning of the test, usually requiring the animal to remain in the lighted area for a period of 30–60 s. (Decker et al. 1990). In this way, all the animals have a similar degree of learning independently of the amount of trials needed to attain it.

PROCEDURE
Mice or rats are generally used. The animals are trained in the same way as in the step-through version. They are placed in the lighted compartment and after they entered with the four paws into the dark area, the door is closed and a mild footshock is delivered. Immediately after the shock they are placed back in the lighted area for another trial. Training would continue this way until the animal remains in the lighted area for a certain period of time (30 or 60 s), a time at which the training is considered to be acquired by all the animals. The number of trials to attain criteria are counted as an indication of the speed of acquisition.

EVALUATION
Retention of the test is measured 24 or 48 Hs later. The animals are placed in the lighted area, the door opened and the latency to step with the four paws into the dark area is recorded. A cut-off latency of 180 or 300 s is usually imposed.

CRITICAL ASSESSMENT OF THE TEST
This modification of the classical inhibitory avoidance procedure is useful to determine the effect of drugs on acquisition as amnesic drugs significantly increase the number of trials to reach the criterion. Saline-treated animals generally need 1–2 trials to reach criterion while animals under diazepam or scopolamine need 3–6 trials. Once the animals have been trained to a predetermined criteria (i.e., 30 s. avoidance) any effect of the drug on the retention trial can be associated to drug effects on consolidation rather on the acquisition process (Brioni 1993; Decker et al. 1990). Alternatively, drugs could be administered before the test session in order to determine the effect of the drug on retrieval processes. Brain lesions differentially affect acquisition or retention (Hepler et al. 1985; Tomaz et al. 1992), and this task has also been used to demonstrate the effect of grafts on recovery of function (Fine et al. 1985).

MODIFICATIONS OF THE TEST
This test can also be performed as a continuous trial-to-criterion. In this procedure the door is not closed after the animal enters the dark area and upon delivery of the shock the animal can escape to the lighted area. The experimenter does not have to touch the animal to placed it in the lighted area for the next trial.

Kameyama et al. (1986) described a similar model for the study of learning and memory in mice using a step-down-type passive avoidance- and escape-learning method.

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F.3.1.6 Scopolamine-induced amnesia in mice

PURPOSE AND RATIONALE

The administration of the antimuscarinic agent scopolamine to young human volunteers produces transient memory deficits (Drachman and Leavitt 1974). Analogously, scopolamine has been shown to impair memory retention when given to mice shortly before training in a dark avoidance task (Dilts and Berry 1967; Glick and Zimmerberg 1972; Schindler et al. 1984). The ability of a range of different cholinergic agonist drugs to reverse the amnesic effects of scopolamine is now well documented in animals and human volunteers. However, the neuropathology of dementia of the Alzheimer type is not confined to the cholinergic system (Iversen 1998).

PROCEDURE

The scopolamine test is performed in groups of 10 male NMRI mice weighing 26–32 g in a one-trial, passive avoidance paradigm. Five min after i.p. administration of 3 mg/kg scopolamine hydrobromide, each mouse is individually placed in the bright part of a two-chambered apparatus for training. After a brief orientation period, the mouse enters the second, darker chamber. Once inside the second chamber, the door is closed which prevents the mouse from escaping, and a 1 mA, 1-s foot shock is applied through the grid floor. The mouse is then returned to the home cage. Twenty-four hours later, testing is performed by placing the animal again in the bright chamber. The latency in entering the second darker chamber within a 5 min test session is measured electronically. Whereas untreated control animals enter the darker chamber in the second trial with a latency of about 250 s, treatment with scopolamine reduces the latency to 50 s. The test compounds are administered 90 min before training. A prolonged latency indicates that the animal remembers that it has been punished and, therefore, does avoid the darker chamber.

EVALUATION

Using various doses latencies after treatment with test compounds are expressed as percentage of latencies in mice treated with scopolamine only. In some cases, straight doses-response curves can be established whereas with other drugs inverse U-shaped dose-responses are observed (Schindler et al. 1984).

CRITICAL ASSESSMENT OF THE METHOD

In spite of the fact that the pathogenesis of primary degenerative dementia (Alzheimer's disease) in man has been only partially elucidated, the scopolamine-amnesia test is widely used as primary screening test for so-called anti-Alzheimer drugs.

MODIFICATIONS OF THE METHOD

Amnesia can also be induced by pretreatment with benzodiazepines (Porsolt et al. 1988).

Nabeshima et al. (1986) studied the antagonism against phencyclidine-induced retrograde amnesia in mice.

Lenègre et al. (1988) investigated the effects of piracetam on amnesias induced by scopolamine, diazepam and electroconvulsive shock.

Yamaoto et al. (1990) studied the effect of drugs on the impairment of working memory produced by scopolamine, ethylcholine aziridinium (AF64A) or cerebral ischemia using a repeated acquisition procedure in a three-panel runway apparatus.

Braida et al. (1998) investigated the short (120 min) and long-lasting (360 min) antagonism of scopolamine-induced amnesia in rats in an eight-arm radial maze by a cholinesterase inhibitor.

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F.3.1.7 Memory impairment by basal forebrain lesions in rats

PURPOSE AND RATIONALE

Memory impairment can be produced by lesions caused by bilateral injections of ibotenic acid into the basal forebrain of rats. Water maze tasks, habituation tasks, passive avoidance tasks with a light/dark compartment apparatus, and the inhibition of the decrease of choline acetyltransferase activity in the cortex can be used to evaluate the effect of drugs (Fuji et al. 1993a).

PROCEDURE

Male Wistar rats weighing 270–310 g are anesthetized with sodium pentobarbital (45 mg/kg i.p.) and placed in a stereotaxic apparatus. Neurotoxic lesions of the basal forebrain are produced by injection of ibotenic acid. An injection needle connected to a 5-µl microsyringe is inserted into the basal forebrain, identified according to the Paxinos and Watson (1986) atlas of rat brain (1.5 mm posterior, 2.8 mm bilateral to the bregma, 7.3 mm below the dura). Ibotenic acid is dissolved in 50 mM Na phosphate buffer at a concentration of 12 µg/ml, and then 0.5 ml (6 µg per side) is infused for 5 min. The injection needle is left in place for an additional 5 min to allow the toxin to diffuse away from the needle tip. One week later, the contralateral side is treated in the same manner. The same procedure is used to administer microinjections of 50 mM Na phosphate buffer into the basal forebrain of sham-operated rats. The lesion sites are mainly distributed in the ventromedial globus pallidus.

Three to 5 weeks after the first lesion, the animals are tested on the acquisition of a task in a Morris water maze (Morris 1981), on a habituation task in a novel situation, and in a passive avoidance task with light and dark compartments. The rats are treated once a day during the experiment.

After the behavioral experiments, the animals are sacrificed for determination of choline acetyltransferase activity in the brain according to Fonnum (1975). The tissue is homogenized (4% w/v) in cold 50 mM Na phosphate buffer (pH 7.4), and Triton X-100 (0.55, v/v) is added to homogenates to ensure enzyme release. To 75 µl of enzyme solution, 125 µl of substrate mixture (0.4 mM [^{14}C]acetyl- Co A (50.6 mCi/mmol), 300 mM NaCl, 50 mM Na phosphate buffer (pH 7.4), 8 mM choline chloride, 20 mM EDTA-2Na, and 0.1 mM physostigmine) is added in a scintillation vial and the mixture is incubated at 37 °C for 30 min. After the incubation, 0.8 ml of cold 50 mM phosphate buffer, 0.5 ml of acetonitrile containing 2.5 mg of tetraphenylborate and 2.0 ml toluene are added to the scintillation vial. The vials are shaken lightly and allowed to stand overnight before radioactivity is determined.

EVALUATION

Data are evaluated by usual statistical means. All analyses are followed by a Bonferroni's test.

MODIFICATIONS OF THE METHOD

Fuji et al. (1993b) studied autoradiographically the influence of drugs on alterations in muscarinic cholinergic receptor [^{3}H]QNB binding induced by basal forebrain lesion in rats.

Harder et al. (1996) performed bilateral transsection of the fornix in young adult marmosets producing a specific pattern of cognitive deficits, notably a lack of ability to recall visuospatial tasks learnt preoperatively, and a deficit in acquiring new visuospatial tasks following transsection. This impairment could be ameliorated by cholinergic agonists and 5-HT$_{1A}$ antagonists.

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F.3.1.8 Ischemia-induced amnesia in gerbils

PURPOSE AND RATIONALE

Impairment of cerebral metabolism induced by reduced blood supply is known to induce cognitive deficits (Gibson et al. 1981). Because of the absence of posterior communicating arteries in the brain of Mongolian gerbils, complete forebrain ischemia can be produced by occluding both common carotid arteries resulting in amnesia (Levine and Sohn 1969; Schindler 1983; Chandler et al. 1985).

PROCEDURE

Male Mongolian gerbils (strain Hoe:Gerk) weighing 50–70 g are anesthetized by i.p. pentobarbital injection. Both common carotid arteries are exposed through a ventral neck incision and occluded for 5 or 10 min with miniature aneurysm clips. In sham-operated controls, the common carotid arteries are exposed but not occluded. Twenty-four hours after occlusion, each animal is placed in the bright part of a light/dark-chambered apparatus for training. After a brief orientation period, the gerbil enters the second, dark chamber. Once inside the second chamber, the door is closed which prevents the animal from escaping, and a 100 V, 2-s foot shock is applied through the grid floor. The gerbil is then returned to the home cage. Twenty-four hours later, testing is performed by placing the animal again in the bright chamber. The latency in entering the second dark chamber within a 5 min test session is measured electronically. The latency compared with sham-operated controls is decreased depending on the duration of ischemia. After drug treatment, an increase of latency in entering the dark compartment indicates good acquisition.

EVALUATION

Using various doses a dose-dependent increase of latency can be found after active drugs, sometimes resulting in inverse U-shaped dose-response curves.

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F.3.1.9 Cognitive deficits on chronic low dose MPTP-treated monkeys

PURPOSE AND RATIONALE

Röltgen and Schneider (1994), Schneider et al. (1990, 1993, 1994a,b) described the effect of chronic low-dose 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure on cognitive functions in monkeys. These animals develop cognitive deficits and difficulties in performing previously learned tasks, such as delayed response, delayed matching-to-sample, delayed alternation, visual discrimination reversal, and object retrieval, as well as dopamine depletions in several brain areas without severe Parkinsonian signs as found after high MPZP doses (Burns et al. 1983), see E.7.1.2. In addition, these animals exhibit other behavioral changes such as decreased task persistence, increased restlessness and decreased attention.

PROCEDURE

Adult Macaca monkeys are trained to perform a delayed response task while seated in a restraining chair placed inside a sound attenuating modified Wisconsin General Test apparatus. The monkey sits behind an opaque screen that when raised allows access to a sliding tray that contains recessed food wells with identical sliding white Plexiglas covers that serve as stimulus plaques that can be displaced by the animal to obtain rewards (raisins). Monkeys are trained to retrieve a raisin from one of the food wells after observing the experimenter baits the well. Right and left wells are baited in a randomized, balanced order. Animals are maintained on a restricted diet during the week and tested while food deprived. Training is accomplished with a non-correction procedure with 0 s delay and progressing to 5 s delay. Animals are trained until performance with a 5 s delay is 90% correct or better for at least 5 consecutive days. Each daily session consists of 25 trials. A response is scored a 'mistake' if the monkey makes its response choice to a well that is not baited with reward. A 'no response' error is scored if the monkey fails to respond to a trail within 30 s.

Once the animals are performing at criterion level, MPTP is administered intravenously in doses ranging from 0.05 mg/kg at the start of the study to 0.20 mg/kg. Animals receive cumulative doses up to 60 mg over periods up to one year.

Pharmacological data are obtained after animals consistently show at least a 15% performance deficit on delayed response. Drugs are administered subcutaneously. Delayed response testing begins 8 min after dug administration. On drug testing days, animals receive drug or saline and are tested for delayed response performance, administered drug (or saline), and retested on the delayed response task.

EVALUATION

Delayed response performance after drug administration is compared with matched control performance obtained on the same day prior to drug administration.

The total number of correct responses as well as the number of mistakes and 'no response' errors are tabulated for each session. Date are then expressed as mean (± standard deviation) performance. All animals serve as their own controls. Statistical analysis consists of analysis of variance, repeated measures design, with post hoc comparisons (Bonferroni *t*-test).

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F.3.2 Active avoidance

Active avoidance learning is a fundamental behavioral phenomenon (Herrnstein 1969; Brush 1971; Campbell and Church 1969; D'Amato 1970). As in other instrumental conditioning paradigms the animal learns to control the administration of the unconditioned stimulus by appropriate reactions to the conditioned stimulus preceding the noxious stimulus. The first stage of avoidance learning is usually escape, whereby a reaction terminates the unconditioned stimulus.

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F.3.2.1 Runway avoidance

PURPOSE AND RATIONALE

A straightforward avoidance situation features a fixed aversive gradient which can be traversed by the animal. The shock can be avoided when the safe area is reached within the time allocated (Munn 1950; Capaldi and Capaldi 1972; Hock and McGaugh 1985).

PROCEDURE

Mice or rats of either sex are used and maintained under standard conditions and handled for several days before the experiment. The same box as used in the step-through model can be used in this experiment. The apparatus is uniformly illuminated by an overhead light source. A loudspeaker, mounted 50 cm above the start-box, serves for presenting the acoustic conditioned stimulus (CS; an 80 dB, 2 000 Hz tone from an audiogenerator). The footshock is employed by the same source as in the step-through avoidance. The animal is allowed to explore the whole apparatus for 5 min. The guillotine door is then closed and the animal is placed into the light starting area. After 10 s the acoustic CS is applied and the door is simultaneously opened. Shock is turned on after 5 s. The CS continuous until the animal reaches the safe area. It is left there for 50–70 s (intertrial interval, ITI) before returned to the same area again. The procedure starts again. The training is continued until the animal attains the criterion of 9 avoidances in 10 consecutive trials. On the next day the procedure is repeated until the same learning criterion is reached. The time needed to reach the safe area is measured.

EVALUATION

The time the animal needs to reach the safe area on both days is measured. In addition, the number of errors (not reaching the safe area) is recorded.

MODIFICATIONS OF THE TEST

Several modifications are described in the literature. The CS also can be a light signal. The number of trials and the criterion can be changed.

CRITICAL ASSESSMENT OF THE METHOD

Same as in step-down method.

REFERENCES

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F.3.2.2 Shuttle box avoidance (two-way shuttle box)

PURPOSE AND RATIONALE

Compared to runway avoidance, shuttle box avoidance (two-way-shuttle-box) is a more difficult task. Since the animal is not handled between trials, the shuttle-box can be easily automated (Capaldi and Capaldi 1972).

PROCEDURE

Rats of both sex are used and maintained under standard conditions. The apparatus used consists of a rectangular box 50×15 cm with 40 cm high metal walls, and an electrifiable grid floor. The box is divided by a wall with a manually or solenoid-operated guillotine door (10×10 cm). into two 25×15 cm compartments. Each compartment can be illuminated by a 20 W bulb mounted in the hinged Plexiglas lids. A fixed resistance shock source with an automatic switch (0.5 s on 1.5 s off) is used. Simple programming equipment provides for automatic delivery of the conditioned stim-ulus (CS) and the unconditioned stimulus (US). The apparatus is placed in a dimly lit room with a masking noise background (white noise) of 60 dB. The animal is allowed to explore the apparatus for 5 min with the connecting door open and the compartment lights switched off. The guillotine door is then closed. After 20 s the light is switched on in the compartment containing the animal, and the door is opened. A tone (CS) is presented and 5 s later the floor shock is applied in the illuminated compartment and continued until the animal escapes to the dark side of the compartment, the connecting door is closed and the shock discontinued. After a variable intertrial interval (ITI; 30–90 s) the light is switched on in the previous dark compartment, the door is opened and the animal is required to cross to the other side. The training is continued until the animal reaches the criterion of 9 avoidances in 10 consecutive trials. Retention is tested at different intervals after the original training by retraining the animal to the same criterion again.

EVALUATION

The time the animal needs to reach the safe area on both days is measured. In addition, the number of errors (not reaching the safe area) are recorded.

CRITICAL ASSESSMENT OF THE METHOD

The task is rather difficult due to the lack of a permanent safe area, lack of a simple instrumental response, presence of a variable aversive gradient and increased weight of emotional factor.

MODIFICATIONS OF THE METHOD

Salmi et al. (1994) described a computer-assisted two-way avoidance conditioning equipment for rats.

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F.3.2.3 Jumping avoidance (one-way shuttle box)

PURPOSE AND RATIONALE

Since a high degree of automation and minimum handling are additional requirements for this model, the obvious solution is a simplified one-way avoidance, allowing for the spontaneous or forced return of the animal to the start. In order to enhance the start-goal distinction a vertical gradient is introduced which requires the animal to perform a discrete response of an all-or-none character, such as the jump, which clearly differs from the more continuous translational movements required in the usual avoidance tasks (Tenen 1966; McKean and Pearl 1968).

PROCEDURE

Rats of both sex are used and maintained under standard conditions. The apparatus used consists of a rectangular box 40×25 cm with 40 cm high metal walls, an electrifiable grid floor and a Plexiglas ceiling. A $12 \times 12 \times 25$ cm opaque plastic pedestrial, mounted onto one of the narrow walls of the box provides the isolated goal area. Flush with the horizontal surface of the pedestal moves a vertical barrier, which can either be retracted to the rear wall of the apparatus to expose the goal area or pushed forward to block access to the goal completely. The animal is placed into the apparatus for 5 min with the goal area exposed (barrier re-

tracted). The barrier is then moved forwards and the goal is blocked for 2 s. The first trial starts by exposing the goal area and applying an acoustic CS (1 000 Hz, 85 dB). Electric shocks – US (1.0 mA; 50 Hz; 0.5 s) – are applied 5 s later (once per 2 s), and continued together with the CS until the animal jumps onto the platform. After 30 s the barrier pushes the animal off the platform onto the grid floor. The sequence is repeated until the criterion of 10 consecutive avoidances is reached. Retention is tested on the next day until the animal reaches criterion.

EVALUATION

The time the animal needs to reach the safe area on both days is measured. In addition, the number of errors (not reaching the safe area) is recorded.

CRITICAL ASSESSMENT OF THE METHOD

In automated procedures extinction is more rapid, especially when short inter test intervals are used.

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F.3.3 Discrimination learning

In the experiments described above the animals have no choice between the conditioned stimuli. They have only one conditioned stimulus. The following examples illustrate the special techniques employed for discrimination among different stimulus modalities. The experiments can be classified either as simultaneous or successive discrimination paradigms. An exhaustive survey of discrimination studies can be found in Gilbert and Sutherland (1969).

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F.3.3.1 Spatial habituation learning[2]

PURPOSE AND RATIONALE

The open-field test utilizes the natural tendency of rodents to explore novel environments in order to open up new nutrition, reproduction and lodging resources (Birke and Archer 1983; Cerbone and Sadile 1994). The rate of exploratory behaviors exhibited in an unfamiliar environment is limited through the inherent necessity to avoid potential dangers. The observed behavior therefore is always a compromise between these conflicting interests and is regulated in part by the momentary physiological needs (Gerlai 1999; Groves and Thompson 1970). Spatial habituation learning is defined as a decrement in reactivity to a novel environment after repeated exposure to that now familiar environment. This reduction in exploratory behaviors during re-exposures is interpreted in terms of remembering or recognition of the specific physical characteristics of the environment. The test can be used to examine short-term spatial memory (within-trial reductions in exploratory activity) and/or long-term spatial memory (between-trial reductions in exploratory activity after a retention interval of 24, 48 or 96 h after the initial exposure) (Lát 1973).

PROCEDURE

The open-field apparatus is a rectangular chamber (rats: $60 \times 60 \times 40$ cm, mice: $26 \times 26 \times 40$) made of painted wood or grey PVC. A 25 W red or green light bulb is placed either directly above or beneath the maze to achieve an illumination density at the centre of approximately 0.3 lx. Masking noise is provided by a broad spectrum noise generator (60 dB). Prior to each trial, the apparatus is swept out with water containing 0.1% acetic acid. Housing room and the testing location are separated and animals are transported to the testing room 30 min before testing. The digitized image of the path taken by each animal is stored and analyzed post hoc with a semi-automated analysis system (e.g. Ethovision, Noldus, The Netherlands or Truscan, Coulbourn Instruments, Allentown, PA). In aged or hypoactive rodents testing is performed during the animals dark phase of day. The rodent is placed on the center or in a corner of the open-field for 5–10 minute sessions (mice: up to 20 min, because of the high basal activity level). The animals are re-exposed to the open-field 24 and 96 h after the initial trial (Thiel et al. 1999; Schwarting et al. 1999).

[2] Contribution by E. Dere.

EVALUATION

The exploratory behaviours registered are: (1) Rearings or vertical activity: the number of times an animal was standing on its hind legs with forelegs in the air or against the wall. (2) The duration of single rearings as a measure of non-selective attention (Aspide et al. 2000) (3) Locomotion or horizontal activity: the distance in centimeters an animal moved.

MODIFICATIONS OF THE TEST

In order to assess emotionality parameters either in interaction with spatial novelty (a single exposure) or in interaction with spatial habituation learning (repeated exposures), the aversivity of the open-field can be varied by increasing the size (inducing agoraphobia) and/or the illumination density (inhibits exploration) of the apparatus (File 1980). Rodents exposed to a big sized brightly lit open field tend to spent more time in the corners or close to the wall and avoid the central part of the apparatus. The emotional behaviors registered are: (1) Corner time: the time spent in the 4 corner squares (rats: 15×15 cm; mice: 6.5×6.5 cm). (2) Wall time: the time an animal spent close to the wall as a measure for thigmotaxis (scanning the walls of the apparatus with the vibrissae). (3) Center time: the time spent in the center of the open field (rats: 20×20 cm; mice: 10×10 cm). (4) Defecation: number of boli deposited. (5) Freezing: the time the animal stays completely immobile except for movements associated with respiratory activity (Hall 1936; Frisch et al. 2000). To assess habituation learning not only by decrements in exploratory behaviors but also via increments, one can place an object in the center of the open-field. The number of entries into and the time spent in the center increases from exposure one to exposure two without affecting other indices of spatial habituation (Dai et al. 1995).

CRITICAL ASSESSMENT OF THE TEST

The open-field paradigm is a well validated, simple and time economical test, which has been widely used to examine the neurobiological foundations subserving spatial memory, general activity and emotionality in rodents with different approaches including: lesions, drugs, electrophysiology, neuroanatomy, and neurogenetics.

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F.3.3.2 Spatial discrimination

PURPOSE AND RATIONALE

In the simplest case of discrimination learning the animal distinguishes between two symmetric stimulus-response sets, the equal probability of which has been changes by differential reinforcement events. Position of the cues with respect to the animal's body defines the CS^+ and CS^-. Usually left-right discrimination is employed, while axial orientation of the body is ensured by the construction of the apparatus (Siegel 1969).

PROCEDURE

Rats and mice of both sex are used and maintained under standard conditions. The apparatus used is usually a simple T- or Y-maze, with an electrifiable grid floor. The last 10 cm of each arm are separated from

the rest of the apparatus by a swing-door which prevent the animal from seeing the food cup or the plastic sheet covering the grid in the goal area. A fixed resistance shock source is connected to an automatically operated switch. In an aversively motivated spatial discrimination learning the animal is trained to escape and/or to avoid foot shocks by always going to the right. Training starts by allowing the animal to explore the apparatus. Then the animal is placed on the start and after 5 s electric shocks (0.5 s, 50 Hz, 1.0 mA) are applied at 3 s intervals. The animals are trained to a criterion. On the following day the animal is retrained to the same criterion. After a 60 min interval the safe goal area is shifted to the other arm of the maze and the discrimination is reversed.

EVALUATION

Errors are scored. An error means that the animal enters the wrong arm with all four legs. During retention the number of trials until the animal makes correct choices are counted.

CRITICAL ASSESSMENT OF THE METHOD

The ecology of rodents makes these animals specially proficient in spatial discrimination learning, which is usually mastered in a few trials. Most of the initial errors are not due to the inability of the animal to remember the correct solution, but rather to its tendency to explore alternative pathways.

MODIFICATIONS OF THE TEST

Barnes (1979) and others introduced the radial maze as a modification of spatial discrimination. This method is now well established and widely used.

Ingram et al. (1994a,b) recommended the Stone 14-unit T-maze to find new pharmacological approaches for cognitive enhancement.

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F.3.3.3 Spatial learning in the radial arm maze

PURPOSE AND RATIONALE

Olton and co-workers have developed a spatial discrimination task for rodents that has been extensively used in learning and memory studies, and that has served as the basic task for one of the most important theories on the role of the hippocampus (Olton et al. 1979; Olton and Samuelson 1976). The rat uses spatial information provided by the distal cues in the room to efficiently locate the baited arms. The radial arm-maze allows the study of spatial reference and working memory processes in the rat. In reference memory procedures, information is useful for many sessions/days and may usually be needed during the entire experiment. On the contrary, working memory procedures have a major temporal component as the information presented in the maze (arms baited) is useful for one session but not for subsequent ones; the rat has to remember the information during a delay interval (min to h). Correct choices in the radial arm-maze are rewarded by food.

PROCEDURE

The apparatus is a wooden elevated eight-arm radial maze with the arms extending from a central platform 26 cm in diameter. Each arm is 56 cm long and 5 cm wide with 2 cm high rails along the length of the arm. The maze is well illuminated and numerous cues are present. Food pellets (reward) are placed at the end of the arms. During the test, rats are fed once a day and their body weights maintained at 85% of their free-feeding weight to motivate the rat to run the maze. Animals are trained on a daily basis in the maze to collect the food pellets. The session is terminated after 8 choices and the rat has to obtain the maximum number of rewards with a minimum number of errors.

EVALUATION

The number of errors (entries to non-baited arms) are counted during the session.

MODIFICATIONS OF THE TEST

Depending on the hypothesis being tested in the radial arm-maze (1) animals can be trained extensively and then receive specific brain lesions (hippocampus, septum or fimbria-fornix); after recovery from the surgery the rats are re-trained to determine the cognitive

ability or the speed of recovery as these lesions severely disrupt processing of spatial information (Olton et al. 1978). (2) In working memory studies animals are forced to obtain reward in specific arms (4 arms), and after a time delay they have to either return to the same arms (win-stay) or to avoid these arms and obtain the food in the rest of the arms (win-shift). (3) Animals are trained to find food in only one arm, and after a delay they are required to return to the same arm. Many versions of the maze exist with differences in the shape, arm-length or the number of arms (12 or 24 arm-mazes), but all of them are appetitively-motivated. An aversively-motivated maze was developed by locating an 8-arm maze in a water tank (Buresova et al. 1985). Extensive modifications on the arm-maze were also developed by Kesner and co-workers and are the basis of the multidimensional attribute theory that characterize mnemonic functions (Kesner 1980, 1986).

CRITICAL ASSESSMENT OF THE TEST

The radial arm-maze has been widely used to determine the neurobiological mechanisms underlying spatial learning in rodents and to evaluate the effect of drugs. The deleterious effects of scopolamine, atropine, ethanol, benzodiazepines, haloperidol, ketamine, PCP, as well as the facilitatory effects of physostigmine, nicotine, pramiracetam, picrotoxin, naloxone have been reported in the literature (Levin 1988). One of the disadvantages of the test is that hypothalamic lesions or the anorectic effect of certain drugs (amphetamine) affect the appetitive nature of the maze and animals do not master the maze for this reason.

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F.3.3.4 Visual discrimination

PURPOSE AND RATIONALE

Vision is better than any other sensory system for the analysis of spatial relationships in the environment of the animal. From the retina to the cerebral cortex, the organization of the visual system ensures processing of visual information according to simple principles, i.e. by fitting the distribution of light over the receptive surface to elementary geometrical concepts and by comparing these patterns with images stored in the memory. Visual pattern recognition is one of the most challenging problems of contemporary neurophysiology and experimental psychology, with significant implications for mathematical and technical modeling of perceptual phenomena (artificial intelligence). For review and details see Grüsser and Klinke (1971) and Sutherland (1969). Experimental studies of pattern discrimination must take into account the visual capability of the given species and present the discriminanda under conditions compatible with light sensitivity and acuity of the eye. The constructions of the apparatus should ensure that the discriminanda are viewed from one optimum distance and for a sufficient period of time. A thorough discussion of the pattern discrimination technique is to be found in Munn (1950).

PROCEDURE

Rats and mice of both sexes are used and maintained under standard conditions. The apparatus consists of a square 10×10 cm start area separated by a Plexiglas sliding door from the choice area, which is connected by swing doors to the goal compartment. The grid floor in the starting and the choice areas is electrifiable. The stimulus (mostly plastic cards 8×8 cm) can be attached to the swing doors. The patterns are black on a white background and have different forms. The apparatus is illuminated by a dim light. The animal is placed into the apparatus with all doors open and allowed to explore it. Then it is placed in the start and after 5 s released by raising the Plexiglas door. After another 5 s, electric shocks (1 mA, 50 Hz, 0.5 s, 1/3 s) are applied until the animal escapes through either of the open doors to the safe goal compartment where it is left for some seconds. As soon as this preliminary step is mastered, the stimulus cards are inserted, the negative door is locked and the grid section in front of this door is electrified. The animal is trained to a criterion. On the next day the animal is retrained to the same criterion and retention is expressed in savings. Another parameter which can be used to evaluate the savings is the cumulative number of errors until the criterion is reached (Thompson 1969).

EVALUATION
The number of correct answers as well as the number of trials until the criterion is reached are counted.

CRITICAL ASSESSMENT OF THE METHOD
The method is time consuming and only useful to address a specific hypothesis.

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F.3.3.5 Spatial learning in the water maze

PURPOSE AND RATIONALE
A task was developed where rats learn to swim in a water tank to find an escape platform hidden under the water (Morris 1984). As there are no proximal cues to mark the position of the platform, the ability to locate it efficiently will depend on the use of a configuration of the cues outside the tank. Learning is reflected on the shorter latencies to escape and the decrease on the length of the path to find the platform. Although rodents can find the platform by using non-spatial strategies, the use of a spatial strategy is the most efficient way to escape and young animals develop the spatial strategy after a small number of trials.

PROCEDURE
Different strains of rats are generally used (Long Evans, Wistar, Sprague-Dawley). The apparatus is a circular water tank filled to a depth of 20 cm with 25 °C water (Brioni et al. 1990; Morris 1984). Four points equally distributed along the perimeter of the tank serve as starting locations. The tank is divided in four equal quadrants and a small platform (19 cm height) is located in the centre of one of the quadrants. The platform remains in the same position during the training days (reference memory procedure). The rat is released into the water and allowed 60–90 s. to find the platform. Animals usually receive 2–4 trials per day for 4–5 days until they escape onto the platform. Well-trained rats escape in less than 10 s.

EVALUATION
The latency to reach the escape platform is measured during the training days. A free-swim trial is generally performed after the training days where the escape platform is removed and the animal is allowed to swim for 30 s. With the help of a video system, the latency to reach the previous position of the platform, the number of annulus crossings as well as the time the rat spent in the training quadrant are measured. Well-trained rats show short latencies, a large number of annulus crossings and bias to the quadrant where the escape platform was located during the training sessions.

MODIFICATIONS OF THE TEST
In the "cue" version of the water maze, the platform is clearly visible over the water and allows the evaluation of the motor and motivational aspects of the rat under study, as the animals should easily find the visible platform in 10–15 s. As circling or other non-spatial strategies can be used to find the platform, alternative measures of learning were developed (Gallagher et al. 1993), like the use of brief probe trials during training (rather than at the end), the use of proximity measures (an index of search error or deviations from the optimal path), and the learning index (proximity measures during weighted probe trials). Buresova and co-workers have developed the use of a collapsible platform to improve the accuracy of the water maze (Buresova et al. 1985). To evaluate working memory processes in the water maze, the rat can be trained to find a new platform position and later its performance is tested after a short delay (2–4 h). A spatial discrimination version of the water maze was also developed (Decker et al. 1992; Morris 1984), where the rat has to discriminate between two similar platforms based on spatial differences (one platform provides a mean to escape while the other sinks upon contact).

CRITICAL ASSESSMENT OF THE TEST
This test allows the researcher to study working and reference memory processes, and to dissociate the memory deficits induced by brain lesions or drug injections from the motor, motivational or sensory deficits. This test has been used to study the effect of drugs on memory (Brioni et al. 1991; Brioni et al. 1990. Decker et al. 1992), the participation of the hippocampus, septum, amygdala and nucleus basalis magnocellularis (Morris et al. 1986; Morris 1989), catecholamine depletions, as well as recovery of function (McNamara and Skelton 1993). It has been particularly sensitive to demonstrate that aged rats exhibit deficits in the acquisition of spatial information when compared to young or middle age controls (Rapp et al. 1987). The major advantages of the water maze over the radial arm-maze are: (1) that animals can be trained

in a significantly shorter period of time (one week) while the arm-maze studies require several weeks of training; (2) intramaze cues like odor trails are eliminated in the pool; (3) large dose-response studies can be conducted in a week's time; (4) the motor or motivational problems in the rat can be detected in a cue trial; and (5) that the animals are not food-deprived during the test. From a theoretical point of view, the water maze is an aversively-motivated task while the arm-maze is appetitively-motivated.

MODIFICATIONS OF THE METHOD

Nitta et al. (1994, 1996; Nabeshima 1995) found an impairment of the performance of the water maze task in rats treated by infusion into the cerebral ventricle for 14 days with β-amyloid protein which consisted of senile plaques of Alzheimer's disease. The authors recommended that β-amyloid protein-treated rats as an animal model for Alzheimer's disease.

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F.3.3.6 Olfactory learning

PURPOSE AND RATIONALE

Odors provide rodents with important information on the environment and the learning of successive olfactory discrimination problems in rats is closely related to the acquisition rules of higher primates. Odor-reward associations are learned in few trials as odors exert more discriminative control over other sensory modalities like tones or lights (Nigrosh et al. 1957). Animals have to learn to discriminate an arbitrary designated positive odor (i.e., banana) from a negative one (i.e., orange) to receive a reward.

PROCEDURE

Rats (Sprague-Dawley or Long Evans) are generally used. In procedures described in the literature (Eichenbaum et al. 1992; Roman et al. 1993), animals are deprived of water for 48 h before the training and during the test they receive ad libitum water for only 30 min. The olfactory apparatus is a rectangular box ($30 \times 30 \times 55$ cm) with a photosensitive cell mounted on top of the water spout/odor outlet. Rats are trained to approach the water spout and to brake the light beam. Responses to the positive odor are rewarded with water while responses to the negative odor results in the presentation of a light flash. The intertrial interval before the presentation of a new odor is usually 15 s. and the sessions last 30 min. per day. Sessions are terminated when the rat makes 90% correct choices or after 400 trials.

EVALUATION

The animal is rewarded with 0.05 ml of water when it brakes the beam to the positive odor or when it does not respond to the negative odor. Incorrect responses ("no go" to the positive odor or "go" to the negative odor are followed by a flash and a longer intertrial interval). Results are reported as the % correct responses or as a logit transformation of the % correct/incorrect response ratio.

CRITICAL ASSESSMENT OF THE TEST

The anatomical connections from the olfactory bulbs to cortical as well as subcortical areas are fairly well

known, and brain lesions that impair olfactory discrimination learning (Otto et al. 1991; Roman et al. 1993; Stäubli et al. 1984) could be used as models of amnesia. Systemic injections of scopolamine disrupts the performance of rats during long delays with no effect on immediate recall, data that are consistent with the effect of scopolamine in humans (Ravel et al. 1992). Antagonists of the NMDA receptor impair acquisition of odor discriminations and block LTP, an electrophysiological correlate of memory (Lynch and Stäubli 1991). The test can also be used to evaluate the cognitive effects of drugs such as ACTH analogs that facilitate the storage of olfactory information (Roman et al. 1989).

MODIFICATIONS OF THE TEST

A T-maze with controlled access to the reward compartment was developed (Ravel et al. 1992), that allows the test to evaluate working memory processes after time delays (1–3 min.).

Willer et al. (1992) examined the effects of a competitive NMDA agonist on the ability of rats to acquire potentiated aversions to the odor element of a taste-odor compound.

Larson et al. (1995) studied in rats the facilitation of olfactory learning by a modulator of AMPA receptors.

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F.3.3.7 Aversive discrimination in chickens

PURPOSE AND RATIONALE

One to 2-days old chicks have been extensively used to study learning and memory. The chicks are trained to discriminate between an aversive and a non-aversive stimulus (colored glass beads) in a single 10–15 s learning trial. Following this learning trial, retention is monitored over a comprehensive range of learning-retention intervals to chart the course and to differentiate possible stages of memory formation. Three behaviorally sequentially dependent stages have been identified: a short-term memory stage, lasting approximately 10 min after learning; an intermediate-memory stage, available between 20 and 50 min post-learning, and a long-term memory stage available from 60 min after learning. The three stages are behaviorally separated by transient retention deficits occurring at around 15 and 55 min post-training. Gibbs and Barnett (1976), Gibbs and Ng (1976, 1979), Rickard et al. (1994), Bennett et al. (1996), Ng et al. (1997) used a three-stage model to test the influence of drugs on memory formation.

PROCEDURE

Day-old, male, White-leghorn black-Australop chickens are housed at an ambient temperature of 28 °C. In a single passive avoidance learning trial, chicks are pre-trained to peck at a 4 mm chromed bead, dipped in water and presented for 10 s. A similar bead, dipped in the chemical aversant methyl anthranilate, is the presented for 10 s. Data from chicks failing to peck the bead on this trial or not showing characteristic disgust responses after pecking are eliminated from subsequent data analysis. Retention is tested by presenting for 10 s a dry bead similar to that used for the pre-training trail.

In a discrimination paradigm, chicks are trained to avoid a red bead and are tested on red and blue beads successively and discrimination ratios registered.

In an additional discrimination paradigm, chicks are given two aversive beads – a chrome and a red bead. They are trained on one of the aversive beads sometime before drug treatment. The other aversive bead is associated with drug treatment and the chicks are subsequently tested with all three beads.

Twenty different chicks are used for each data point. Retention in both paradigms is indexed by the proportion of chicks avoiding the aversive bead, with the proviso that in the discrimination paradigm is indicated by the avoidance of the red bead and pecking on the blue bead. Retention is tested at various times between 5 and 180 min after learning or at 24 h.

Drugs are administered in 0.1 ml volumes subcutaneously into a fold of skin on the ventral side of the rib cage.

EVALUATION

Discrimination ratios of treated animals after various time intervals following administration of drugs are statistically compared with saline treated controls.

CRITICAL ASSESSMENT OF THE METHOD

The test has been used with slight modifications successfully by many authors in several research groups (e.g., Hölscher and Rose 1992; Sandi and Rose 1994; Stephenson and Andrew 1994; Zhao et al. 1994; Deyo and Hittner 1995; Crowe and Shaw 1997; Venero and Sandi 1997; Bennett et al. 1998; Tiunova et al. 1998; Rose and Stewart 1999)

MODIFICATIONS OF THE TEST

Bourne et al. (1991) compared the taste and odor aversant methyl anthranilate with the odorless quinine as averants.

Clements and Bourne (1996) and Stamatakis et al. (1998) injected drugs, e.g., GABA agonists or α_2-noradrenergic agonists and antagonists, directly into the intermediate median hyperstriatum ventrale of day-old chicks, prior to training on a chrome bead dipped in either methyl anthranilate or quinine.

Colombo et al. (1997) injected enkephalins into 2 regions of the 2-day-old chick brain: the intermediate medial hyperstriatum and the lobus parolfactorius.

Tiunova et al. (1996) trained chicks to discriminate between edible chick crumbs and arrays of colored bead glued to the floor of their cage.

Gilbert et al. (1989) reported a simple, one-trial, learning paradigm in young chicks. Chicks were separated from their brood mates and placed in a small isolation chamber. A T-maze connected the isolation chamber to the brood space, allowing the chick to escape isolation stress and rejoin the brood. When the chick successfully negotiated the corridor, the latency to perform this task was recorded. On a subsequent trial, any improvement in the speed of performance was recorded to reflect the chick's memory of the task.

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F.3.4 Conditioned responses

F.3.4.1 Conditioned nictitating membrane response in rabbits

PURPOSE AND RATIONALE

The rabbit's classically conditioned eyeblink response has become a widely used model system for studying associative learning in mammals (Gormezano et al. 1987) and to find drugs potentially useful in the treatment of age-related memory disorders (Ghoneim et al. 1994; Woodruff-Pak et al. 1994a,b, 1997; Solomon et al. 1988, 1995a,b).

PROCEDURE

Animals

New Zealand white albino rabbits weighing 2.0 kg are housed individually under consistent light with free access to food and water.

Apparatus and general procedure

A small loop of surgical nylon is sutured into the right nictitating membrane, and the surrounding hair is removed. One day later, the rabbit is placed in a Plexiglas restrainer, and two stainless-steel wound clips are applied to the skin over the parietal region. The rabbit is fit with a headmount that supports a photoresistive assembly for recording the nictitating membrane response by physical coupling with a length of thread to the nylon loop in the nictitating membrane. The transducer assembly converts nictitating membrane movements into elec-trical signals that are subjected to an analog-to-digital conversion using a 5-ms sampling rate and a resolution of 0.06 mm actual membrane extension. The animal is then positioned in a ventilated, sound-attenuated chamber facing a stimulus panel containing an 11.4-cm speaker and two 6-W, 24-V DC house lights, one mounted at each side of the speaker. During the course of the experiment, two stimuli are employed as conditioned stimulus: a) a 1000-ms, 1-kHz, 84-dB tone; b) a 1000-ms, intermittently presented light produced by interruption of the house lights at 10 Hz to yield a change in illumination, measured at the eye level of the rabbit from 32.11 to 8.01. The unconditioned stimulus is a 100-ms, 3-mA, 60Hz shock delivered to the wound clips by a constant current shock generator.

Drugs

Drug solutions or saline are injected subcutaneously into the cervical area of the rabbit via an infusion pump at a rate of 3 ml/min, 30 min before behavioral testing.

Procedure

Experimentally naive rabbits are random-ly assigned in equal numbers to each of the treatments ($n = 10$ per treatment). The experiment consists of two phases: Phase 1 is an adaptation day followed by 9 days of acquisition training. No stimuli are presented during the 60-min adaptation session. Subjects are injected with their assigned treatment 30 min before each acquisition session. Each acquisition session consists of 30 tone-shock and 30 light-shock trials presented in a randomized sequence within 10 trial blocks, with the restriction that no more than three consecutive tone or light trials can occur. On each conditioned stimulus – unconditioned stimulus trial, the offset of the 1 000-ms tone or light conditioned stimulus occurs simultaneously with the onset of the 100-ms unconditioned stimulus. The inter-trial interval is about 60 s. A response is defined as at least a 0.5-mm extension of the nictitating membrane. Responses occurring during the tone or light conditioned stimulus, but before the unconditioned stimulus are recorded as conditioned response; those occurring after the unconditioned stimulus onset are recorded as unconditioned response.

EVALUATION

The data are analyzed by repeated measures analyses of variance and Tukey tests. The significance level is set at $p < 0.05$.

CRITICAL ASSESSMENT OF THE METHOD

The rabbit nictitating membrane response has been used widely to study the effects of drugs on learning (Schindler and Harvey 1990).

MODIFICATIONS OF THE TEST

Several authors used a corneal airpuff as unconditioned stimulus (Robinson et al. 1993; Solomon et al. 1995, 1996).

Scavio et al. (1992) studied post-training effects of several drugs, such as amphetamine, chlorpromazine, ketamine, and scopolamine, on the acquisition and extinction of the rabbit's conditioned membrane response.

The effects of modulating tone frequency, intensity, and duration on the classically conditioned rabbit nictitating membrane response were studied by Kehoe et al. (1995).

Solomon et al. (1996) described behavioral paradigms for delay, trace, and long-delay conditioning.

Various drugs influence the nictitating membrane response in rabbits, such as morphine (McEchron and Gormezano 1991), cocaine (Marshall-Goodell and Gormezano 1991), sodium pentobarbital (Chen et al. 1992), haloperidol (Sears and Steinmetz 1990), MDA (3,4-methylenedioyamphetamine) (Kirkpatrick-Steger

et al. 1991; Romano and Harvey 1993), harmaline (Harvey and Romano 1993; Du and Harvey 1997), ketamine (Ghoneim et al. (1994), isoflurane (El-Zahaby et al. 1994), L-NAME (nitric oxide synthesis inhibitor, Du and Harvey 1996), serotonin antagonists and agonists (Welsh et al. 1998a,b), calcium antagonists (Woodruff-Pak et al. 1997), nitrous oxide (Ghoneim et al. 1999).

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F.3.4.2 Automated learning and memory model in mice

PURPOSE AND RATIONALE

Vanover and Barrett (1998) developed and validated pharmacologically an automated, relatively rapid, and reproducible behavioral model of learning and memory using an autoshaped procedure in mice. Autoshaping procedures were first described for pigeons by Brown and Jenkins (1968) and have been used subsequently as a means to assess learning and memory in monkeys (Sidman and Fletcher (1968), in rats (Steckler et al. 1993; Oscos et al. 1998), and in mice (O'Connell 1980).

PROCEDURE

Naive male mice weighing 25–35 g are randomly assigned to experimental groups. They are housed at approximately 22 °C at a 12 : 12 light/dark cycle with food and water continuously available. Twenty-four hours before each test, mice are isolated into individual cages and all food is removed. Water remains continuously available. Tests are conducted on two consecutive days. Mice are fed 1.5 g food immediately after the first session (day 1).

For experimental sessions, mice are placed in sound-attenuating enclosures containing specially designed operant chambers ($12.5 \times 11 \times 12.5$ cm) equipped with a recess (dipper well; 2.2 cm diameter, 1.3 cm deep) for dipper accessibility on one wall of the chamber and two additional smaller holes (1.3 cm diameter, 0.9 cm deep) on either side of the dipper well. The dipper can be raised into the dipper well for a sucrose solution (110 g/1.0 l water) reinforcer presentation. Each recess has a photocell monitoring nose-poke responses. In addition, a speaker capable of sounding a stimulus tone is located on the wall opposite to the dipper and a house-light is on the ceiling. A computer and associated interface controls stimulus events and records nose-poke responses.

Five to 9 mice per group are tested in a 2-day procedure designed to measure acquisition and retention of nose-poke response under an autoshaping schedule of reinforcement. Experimental sessions are conducted at the same time each day for 2 consecutive days. Both the acquisition session (day 1) and the retention session (day 2) are identical.

During the autoshaping procedure which is used to measure acquisition and retention of the response reinforcement for nose-poking a tone is added. This tone sounds on a variable-interval schedule of presentation (mean of 45 s, range 4–132) and stays for either 6 s or until a nose-poke in the dipper well is made before the end of the 6 s period, at which time the tone is turned off and a dipper with sucrose solution is presented. If a mouse fails to make a dipper well nose-poke during the tone, the dipper is automatically presented at the termination of the tone. A dipper well nose-poke response made during the presence of the tone is counted as reinforced response. Dipper well nose-poke responses made while the tone is off are counted but have no consequence. Each sessions lasts for 2 h or until 20 reinforcers have been earned, whichever comes first. In addition to dipper well nose-poke responses, nose-pokes in the smaller holes to the left and right of the dipper are counted but have no consequence.

Drugs in various doses or vehicle are administered i.p. immediately before the session.

Rates of nose-poke responses made in the small holes on either side of the dipper well serve as a measure of general activity. The computer presents tones on a variable interval with a mean of 45 s and 10 possible intervals (4, 10, 15, 22, 29, 38, 49, 64, 87, 132 s) chosen randomly without replacement until all intervals have been used.

The latency of the response to the 10^{th} reinforcer is considered as a measure of acquisition and retention, because all mice have been exposed to all possible intervals (in random order) by the 10^{th} tone. In order to eliminate the variance due to the variability of the first reinforcer, it is necessary to adjust any measure of acquisition and retention according to the response latency to the first reinforcer. The 10^{th} response latency measure is thus adjusted by subtracting the response latency to the first reinforcer (L-10−1) in order to ensure all mice have been exposed to the reinforcement contingency.

An other measure of acquisition and retention consists of the rate of nose-poke responding in the dipper well during the total session.

EVALUATION

The mean and standard error of the mean are calculated for every group. Two-tailed Student *t*-tests are used to compare two independent groups on any one dependent variable. In addition, two-tailed paired *t*-tests are conducted to compare data from day 1 to data from day 2 for any one group. One way analyses of variance (ANOVA) are conducted across groups for effects of dose on acquisition (day 1 performance). One way analyses of covariance (ANCOVA) are conducted across groups for effects of dose for retention (day 2 of performance) with the performance on day 1 as the covariate. When an ANOVA or ANCOVA shows statistical significance, post-hoc Duncan's multiple range tests are conducted with every group compared with vehicle. *P* values less or equal to 0.05 are considered statistically significant.

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F.3.5 Studies in aged monkeys

PURPOSE AND RATIONALE

Nonhuman primates have the closest taxonomic relationship to humans, sharing many morphologic and physiologic similarities in the central nervous system. These similarities increase the likelihood that studies in aged nonhuman primates will provide information about drugs that is relevant to humans. Nonhuman primates offer additional advantages for neurobehavioral animal models of aging in that many of the behavioral processes thought to be affected by aging (e.g. reaction time, attention, learning and memory) can be studied easily in nonhuman primates. Evidence is beginning to accumulate suggesting that certain neurological and behavioral deficits are observed in aged human and in nonhuman primates (Bartus 1979; Struble et al. 1982; Wisniewski et al. 1973).

PROCEDURE

The apparatus developed specifically for the series of studies used to develop the primate model was the Automated General Experimental Device (AGED), the rationale upon which its development was based and its application to geriatric research have been discussed in detail elsewhere (Bartus 1979; Bartus and Dean 1981; Dean et al. 1983). The AGED is a totally automated, computer-controlled testing system, whose prominent feature consists of a 3×3 matrix of stimulus response (SR) panels. Each SR panel is hinge-mounted directly in front of the reinforcement well so that when a panel is pushed, a red switch is magnetically activated and a reinforcement well is exposed. Both colored and patterned stimuli can be projected onto the SR panels. A plastic partition with a stimulus window and armholes separates the monkey from the SR matrix. The stimulus observation window is equipped with a photocell and an infrared light source to detect when the monkey's head is oriented toward the stimuli.

EVALUATION

The monkey must remember the stimulus location to get a reinforcement. Number of correct answers will be counted as well as the time until the monkey answers correctly.

CRITICAL ASSESSMENT OF THE METHOD

This system provides an accurate and objective means of collecting data under a number of behavioral paradigms. Further, this system provides experimental control over a number of variables that might confound behavioral measures (especially in drugged or aged subjects), thus simplifying the interpretation of differences observed and increasing the likelihood that these interpretations are accurate.

MODIFOCATIONS OF THE METHOD

Cai and Arnstein (1997) described dose-dependent effects of Dopamine D1 receptor agonists on spatial working memory in aged monkeys.

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F.3.6 Electrophysiological methods

F.3.6.1 Long-term potentiation in hippocampal slices

PURPOSE AND RATIONALE

Long-term potentiation (LTP) in the hippocampus is perhaps the most dramatic example of activity-dependent synaptic plasticity that has yet been identified in the mammalian brain (Landfield and Deadwyler 1988; Bashir et al. 1994). A brief tetanus to any one of a number of monosynaptic excitatory pathways in the hippocampus can enhance the amplitude of evoked responses in the tetanized pathway for h or days thereafter. The fact that it occurs in the hippocampus has done much to stimulate interest in LTP as a synaptic model of memory, since the importance of the hippocampus for memory processing has been evident ever since the discovery that its bilateral removal in man causes a profound impairment in the ability to lay down new memories (Scoville and Milner 1957; Milner 1972).

The particular popularity of the slice preparation prepared from the rodent hippocampus rests on its lamellar and laminar organization (Teyler 1980).

PROCEDURE

Transverse slices, 400 mm thick, are cut from the hippocampus of male albino guinea pigs weighing 250–300 g and prepared for electrophysiological recordings (Teyler 1980; Tanaka et al. 1989, 1990). Slices are incubated for 90–120 min in the recording chamber to allow equilibration with artificial cerebrospinal fluid. They are submerged, placed on a nylon mesh and perfused at a flow rate of 2–2.5 ml/min with oxygenated (95% O_2/5% CO_2) cerebrospinal fluid having the following composition (in mM): NaCl 124, KCl 3.3, $CaCl_2$ 2.5, KH_2PO_4 1.25, $MgSO_4$ 2, $NaHCO_3$ 25,7, glucose 10. The recording chamber is maintained at 33 ±2 °C. The extracellular population spike is obtained using glass microelectrodes filled with 2 M NaCl, which have resistances of 2–5 MΩ. The electrodes are placed into the stratum pyramidale of CA1 or CA3. The signal is amplified and stored on magnetic discs for later analysis. The evoked responses are averaged and analyzed off-line using a personal computer. The magnitude of the population spike is evaluated by taking the voltage difference between the negative peak and the following positive peak. Either mossy fibers in the hilus fasciae or commissural/associational fibers in the stratum radiatum are activated via bipolar, sharpened silver wire electrodes insulated except for the tips. Constant current pulses (100 ms) are delivered with a frequency of 0.2 Hz, only during the test intervals. The stimulation intensity is adjusted to elicit the population spike of about 40% and 80% of its maximal amplitude in CA1 and CA3, respectively. After the baseline is recorded for 10–20 min, LTP is induced by repetitive stimulation of 100 pulses at 20 Hz for 5 s in CA1 and at 50 Hz for 2 s in CA3 at the same strength as for the test pulses. Responses by test pulses are recorded 0, 10, 20 and 30 min after repetitive stimulation. The test drugs are dissolved in the artificial cerebrospinal fluid and applied extracellularly at various concentrations by switching perfusion reservoirs.

EVALUATION

The time course of LTP is registered for CA1 and CA3. The mean percent increase in the amplitude of the population spike from baseline responses after drug application is compared with controls.

MODIFICATIONS OF THE METHOD

Fujii et al. (1997) studied the effects of an adenosine A_1 receptor antagonist, 8-cyclopentyltheophylline, on the reduction of long-term potentiation in CA1 neurons of **guinea pig** hippocampal slices. Reduction of long-term potentiation (depotentiation) was achieved by delivering a train of low-frequency afferent stimuli 20 min after the tetanus.

Behnisch and Reymann (1993) employed slices of hippocampal area CA1 in the **rat** to test the hypothesis that the activation of metabotropic glutamate receptors during tetanization is necessary for the maintenance of long-term potentiation.

Akhondzadeh and Stone (1995) studied the phenomenon of hippocampal long-term depression. Extracellular recordings were made in the CA1 pyramidal cell layer of **rat** hippocampal slices following orthodromic stimulation of Schaffer collateral fibres in stratum radiatum.

Oomura et al. (1996) measured long-term potentiation in hippocampal slice preparations after a brief tetanic stimulation at the Schaffer collateral/-commissural afferents of senescence-accelerated **mice** which were treated from 3 weeks of age up to 10 months either with subcutaneous injections of acidic fibroblast growth factor or saline.

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F.3.6.2 Long term potentiation *in vivo*

PURPOSE AND RATIONALE

Placing recording and bipolar stimulating electrodes in the granule cell layer of the dentate gyrus and angular bundle allows the evaluation of long-term-potentiation *in vivo*, even in freely moving animals, and the comparison with effects on learning.

PROCEDURE

Female Sprague Dawley rats weighing 225–250 g are anesthetized with 1.5 g/kg urethane (i.p.) and placed in a Kopf stereotaxic instrument. The recording and bipolar stimulating electrodes are placed in the granule cell layer of the dentate gyrus and angular bundle, respectively (from bregma and cortical surface: Dentate: A.P. –4.0 mm, M.L. +2.4 mm, D.V. 3.0 mm. Angular bundle: A.P. –7.9 mm, M.L. +4.0 mm, D.V. –3.0 mm, incisor bar –5.0 mm). The recording electrodes are pulled from thin-walled glass capillary tubes, filled with 150 mM NaCl, and adjusted to resistances ranging from 2.0 to 4.0 MΩ. Stimulating electrodes are made from twisted nichrome wire with Teflon insulation, and approximately 0.75 mm separated each tip. The recording electrode is first lowered into area CA1 of the dorsal hippocampus. The stimulating electrode is then placed into the ipsilateral angular bundle. As the recording electrode is lowered further, field potentials are evoked to determine when the recording electrode enters the dentate granule cell layer.

All rats are maintained at 37 °C. Responses are evoked using a Grass S-88 stimulator and a Microprobe System M-7 070A amplifier, and are recorded on a Nicolet 310 oscilloscope.

Once the electrodes are appropriately placed, field potentials are generated over a range of stimulus intensities to generate an input/output (I/O) curve (pulse duration = 1 ms). The field potentials are quantified in two ways: The population spike (PS) is expressed as the distance from the deepest point (in mV) of negativity to the preceding highest positivity on the left and right side of the response, and then averaged. The slope of the rising phase of the population excitatory postsynaptic potential (pESP) is measured in mV/ms. The amplitude of the test pulse is based is based on the I/O curve being 25% of the current intensity which evokes the maximal population spike. Long-term-potentiation is induced using three theta-burst stimulus trains, each delivered 1 min apart. Each train consists of five groups of four pulses at 100 Hz, separated by an interval of 150 ms. Each train is delivered at the stimulus intensity which evokes the maximal population spike at the I/O curve.

At the end of the experiment, the electrode placements are verified using standard histological techniques.

EVALUATION

Student's *t*-tests are used to assess the significance of differences between the means of both the population spike amplitudes and the pESP slopes.

CRITICAL ASSESSMENT OF THE METHOD

Hölscher et al. (1997a,b) argued that high-frequency stimulation-induced long-term potentiation and low-frequency stimulation-induced depotentiation in area CA1 of the hippocampus are not good models for learning.

MODIFICATIONS OF THE METHOD

Errington et al. (1987) perfused a NMDA receptor antagonist through a push-pull cannula into the dentate gyrus of anesthetized **rats** in order to observe its effect on the induction and maintenance of long-term potentiation and on the increase in release of endogenous glutamate associated with long-term potentiation.

Bennett et al. (1992) recommended cytochrome oxidase inhibition as an animal model of Alzheimer's disease. Rats were infused chronically with the selective inhibitor of cytochrome oxidase, sodium azide, delivered via subcutaneously implanted minipumps. The azide treatment impaired both spatial and non-spatial learning. Further, the azide treatment inhibited a low-threshold form of hippocampal long-term potentiation, primed burst potentiation.

Croll et al. (1997) used this model to study time course and corresponding pathology of learning deficits in rats.

Namgung et al. (1995) described the characteristics of long-term potentiation in the intact **mouse**. Perforant

path stimulation evoked both a population excitatory postsynaptic potential and a population spike potential from the hippocampal dentate gyrus in urethane anesthetized animals. Long-term potentiation, as measured by increased population-spike amplitude and population excitatory postsynaptic potential slope, was successfully induced and reliably maintained at a stable level for at least 12 h.

Davis et al. (1997) described a simple method for inducing and monitoring long-term potentiation at perforant path-granule cell synapses in the dentate gyrus of freely moving mice using readily available miniaturized components. Tetanic stimulation induced long-term potentiation of the field excitatory postsynaptic potential and the population spike which persisted for more than 24 h but was not present 10 days after the tetanus.

Jibiki et al. (1993) and Kubota et al. (1994) studied the drug-induced blockade of induction of long-term potentiation in perforant path-dentate gyrus pathway in chronically prepared **rabbits**.

Gutnikov and Gaffan (1996) studied the effects of a NMDA receptor antagonist on memory acquisition and retrieval of visual-reward associations in the object-in-place memory task and on NMDA neurotoxicity in **rhesus monkeys**.

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F.3.6.3 Long latency averaged potentials

PURPOSE AND RATIONALE

Wirtz-Brugger et al. (1986, 1987) studied long latency averaged evoked potentials (P300) in anesthetized rats as a possible model for detecting memory-enhancing drugs. The P300 waveform is described as a positive long-latency (~300 ms) potential believed to reflect endogenous cognitive processes rather than exogenous physical parameters of the stimulus. Compounds that have been shown clinically to enhance cognitive ability also significantly increase the integrated area under the P300 wave.

PROCEDURE

Male Wistar rats weighing 300–500 g are anesthetized with 120 mg/kg i.p. Inactin®. Stainless steel screws serve as recording electrodes located at the surface of the cortex of seven distinct brain areas: posterior: Pz (midline), P3, P4 (lateral); central: Cz (midline); and frontal: Fz (midline, F3, F4 (lateral). A linked reference is provided with platinum needle electrodes behind the ears. All leads are fed into a digital averaging computer. Event-related potentials are elicited in response to an oddball paradigm of two tones (500 Hz frequent and 3 KHz rare) randomly presented with a probability of 10% for the rare tone. The intensity of the tones is 95 bB, pulse duration 100 ms, rise/fall 9.9 ms. The auditory stimuli are delivered bilaterally at a rate of 0.3/s through special Nicolet tubal tip inserts into the ear of each subject. One repetition of the paradigm consists of 300 tones. It is presented twice, before and after drug application. Evaluation of the rare responses consists of defining and comparing the P300 in terms of integrated area before and after drug. P300 area is calculated by integration of area under the curve.

EVALUATION
Values are expressed as means and standard error per group and percent changes from control. Statistical evaluation consists of paired t-tests to demonstrate significant differences.

MODIFICATIONS OF THE METHOD
Caudle (1993) demonstrated long latency potentials in the CA1 regions of the rat hippocampal slice suggesting that the *in vitro* P3 is an *in vitro* version of the long latency evoked potential known as the P300 in electroencephalogram studies.

Ikeda et al. (1995) studied the effect on a nootropic compound on event-related potential P300 in rats with lesions of the nucleus basalis magnocellularis.

Antal et al. (1994) studied the influence of cholinergic agents on visual discrimination and P300 in the behaving **monkey**. P300 latency was increased following scopolamine administration. Acetyl-L-carnitine increased P300 amplitude and decreased its latency.

In **squirrel monkeys**, clonidine significantly decreased the area and increased the latency of P300-like potential (Swick et al. 1993).

Kaga et al. (1992) studied P300 and choline acetyltransferase immunohistochemistry in septohippocampal neurons of **cats**.

Wang et al. (1999) reported that P300-like potential depends on muscarinic receptor activation in **rabbits**.

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F.3.7
Metabolic influence

F.3.7.1
Sodium nitrite intoxication ($NaNO_2$)

PURPOSE AND RATIONALE
The manipulation of brain metabolism was used to show the beneficial effects of substances which influence learning and memory. Gibson et al. (1976, 1978), during investigations of sodium-nitrite ($NaNO_2$) on brain metabolism, demonstrated a close relationship between oxidative metabolism and cholinergic function. From the results of their studies, the possibility cannot be excluded that an induction of impairment of the cholinergic transmission in addition to a deficiency in brain metabolisms was induced (Schindler et al. 1984).

PROCEDURE
Male or female mice are used and maintained under standard conditions. Chemical hypoxia is induced by the injection of sodium nitrite ($NaNO_2$: 250 mg/kg s.c.) which reduces the oxygen-carrying capacity of the blood by converting hemoglobin to methemoglobin. This lethal dose (lethality 100% of controls) is injected 60 min after drug treatment. Immediately after the $NaNO_2$-injection the animals are placed in small Makrolon® cages and the time between injection of $NaNO_2$ and cessation of respiration is recorded (Hock 1993).

EVALUATION
The time between injection of $NaNO_2$ and cessation of respiration is recorded. The prolongation of survival time is expressed in percent.

MODIFICATIONS OF THE METHOD
Using lower doses of $NaNO_2$ mice are submitted to a positive reinforcement paradigm (Schindler et al. 1984). Groups of 10 mice are water deprived for 24 h. The mice are treated with the test compound 45 min before they are placed individually in a large chamber. On one wall of the chamber, there is a small compartment that contains a water bottle. The mouse easily finds the bottle and is allowed to drink for 30 s. Each mouse then receives a subcutaneous injection of 75 mg/kg $NaNO_2$ before being returned to the home cage. Twenty-four hours later, retention testing is performed by placing the mouse in the large chamber, but at this time, the small compartment is kept empty. The duration and frequency of the mouse's exploration of the small compartment while searching for water is evaluated over a period of 3 min. An increase in duration and frequency correlates with improved learning

The tight rope test (Barclay et al. 1981) was used to test the effect of $NaNO_2$ with and without treatment on performance in mice (Peterson and Gibson 1982; Gibson et al. 1983).

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Chapter G
Effects on peripheral nerve function

G.1
Local anesthetic activity

G.1.0.1
General considerations

One has to generally distinguish between conduction anesthesia, infiltration anesthesia, and surface anesthesia (Fromherz 1922; Schaumann 1938), and special pharmacological tests have been developed for each of these.

The mode of action of local anesthetics has been reviewed by Ritchie and Greengard (1966), Ritchie (1971), Borchard (1977), Steiner (1978), Butterworth and Strichartz (1990).

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G.1.1 Conduction anesthesia

G.1.1.1 Conduction anesthesia in the sciatic nerve of the frog

PURPOSE AND RATIONALE

Based on earlier studies by Sollmann (1918), Fromherz (1922), Fußgänger and Schaumann (1931), and Bülbring and Wajda (1945) on plexus anesthesia in frogs, Ther (1953a,b, 1958) described a method for conduction anesthesia of the sciatic nerve in frogs.

PROCEDURE

Frogs (*Rana temporaria*) of either sex are used and are kept at 4 °C. The frog is decapitated with a pair of scissors. The skin is incised in the thigh region at both sides and the sciatic nerves are carefully exposed in the thigh, avoiding any stretching and injury of the nerve. The frog is suspended on a vertical board. Small pieces of white cotton are soaked with different concentrations of the test preparations (between 0.05% and 1%) or the standard and placed gently around the sciatic nerve for 1 min. Then the cotton swab is removed and the frog is placed with its extremities into a bath with 0.65% NaCl solution. This allows testing for duration and reversibility of the local anesthetic effect. One side is used for the test preparation and the other for the standard (e.g., 0.25% butanilicaine). Every 3 min the frog is removed from the bath and the toes of the legs or the ankle joint are pinched three times with a small forceps. The reflex contraction is abolished when conduction anesthesia is effective. The stimuli are repeated every 3 min until anesthesia vanishes. Two to 5 frogs are used for each concentration.

EVALUATION

Time of onset and duration of anesthesia are recorded for each concentration. Time-response and dose-response curves can be established.

MODIFICATIONS OF THE METHOD

In the original method by Bülbring and Wajda (1945) the frogs were decapitated and the upper part of the spinal cord was destroyed down to the level of the third vertebra. The viscera are removed exposing the lumbar plexus without damaging it. The frog was pinned to a vertical board and the solution of the local anesthetic dissolved in 0.7% saline is put into the pocket formed by the lower abdomen. Different concentrations of HCl are used as stimuli into which the feet of the frog were immersed every min.

Idänpään-Heikkilä and Guilbaud (1999) used a **rat** model of trigeminal neuropathic pain where the neuropathy is produced by a chronic constriction injury of the infra-orbital branch of the trigeminal nerve, and studied the effects of various drugs on this purely sensory model of neuropathic pain (see H.1.2.11)

For mechanical stimulation, a graded series of ten of von Frey filaments with a bending force between 0.217 and 12.5 g was used. The stimuli were applied within the infra-orbital nerve territory, near the center of the vibrissal pad, on the hairy skin surrounding the mystacial vibrissae. Local injection of an local anesthetic (articaine) into the rostral orbital cavity of the lesioned side, into the close proximity of the ligated infra-orbital nerve increased the mechanical threshold to the upper level. The duration of the effect was dose-dependent.

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G.1.1.2 Conduction anesthesia in the sciatic nerve of the rat

PURPOSE AND RATIONALE

Based on earlier studies (Truant 1958; Truant and Wiedling 1958/59, Åström and Persson 1961) Camougis and Takman (1971) have written a detailed description for testing conduction anesthesia of the sciatic nerve in the rat.

PROCEDURE

Male Wistar or Sprague Dawley rats weighing 125 to 175 g are used. The animal is suspended in a prone position by grasping the base of the tail and thoracic cage. A hind limb is extended to its full length and the depression for needle insertion is located by palpation with the left index finger. The site of injection is the area under the skin at the junction of the biceps femoris and the gluteus maximus muscles. The sciatic nerve is blocked in the midthigh region with 0.2 ml of the drug solution administered by a 24- to 25-gauge needle attached to a 0.25 ml tuberculin syringe. Usually a 1% solution of the test drug in 0.9% NaCl is used as a test solution. The other leg is used for a control drug (e.g., procaine or lidocaine). Immediately after the injection, repeated checks of the digit of the foot and the walking behavior are performed. In the normal foot, the digits are wide apart, while in the blocked leg the digits of the foot are close together. Also the successful block is evidenced by dragging of the leg and an ina-bility of the animal to use the leg in walking up the inclined wire mesh cover of the cage. After the time of block for each leg is noted, each animal is examined every 5 to 10 min in order to note the time of recovery.

EVALUATION

From the data, averages for onset and duration of action are calculated, plus the frequency of blocks are noted. Using various doses of test compound and standard, dose-response curves can be established and potency ratios calculated.

MODIFICATIONS OF THE METHOD

With a similar technique Lembeck (1953) tested the effect of the added vasoconstringent agents adrenaline, noradrenaline and Corbasil® to procaine.

Luduena and Hoppe (1952) used guinea pigs to test sciatic nerve block by local anesthetics.

Siems and Soehring (1952) described a model in **guinea pigs** resembling peridural and paravertebal anesthesia in man.

Sciatic nerve blockade in the **rat** was used by Feldman and Covino (1988) to study comparative motor-blocking effects of bupivacaine and ropivacaine.

Grant et al. (1992) used a rat sciatic nerve model for independent assessment of sensory and motor block induced by local anesthetics. Motor block was assessed by measuring hindpaw grip strength with a dynamometer. Sensory block was determined by measuring hindpaw withdrawal latency from radiant heat.

Åkerman et al. (1988) used sciatic nerve block and brachial plexus block in **guinea pigs** for primary evaluation of a local anesthetic.

Rosenberg and Heinonen (1983) found a differential sensitivity of A and C nerve fibres to long-acting local anaesthetics.

The Local Anesthetics for Neuralgia Study Group (1994) designed a surgically implantable nerve irrigation system for intermittent delivery of local anesthetics and evaluated long-term performance and histocompatibility in rats.

Leszczynska and Kau (1992) used a sciatic nerve blockade method in **mice** to differentiate drug-induced local anesthesia from neuromuscular blockade. The drugs were injected into the popliteal space of the right hind-limb. A positive local anesthetic activity was recorded when a mouse was only able to walk using three limbs on an inverted wire mesh screen and the injected limb was hanging in the air. A positive neuromuscular blockade was recorded when a mouse could walk normally on the top of the wire mesh screen but was unable to stay on the inverted screen.

Åkerman et al. (1988) used **guinea pigs** to determine brachial plexus block. The syringe was directed towards a line between the head of the humerus and the manubrium of the sternum and the first ribs. The needle was inserted about 1.5 cm into the pocket felt as a depression by palpation between the head of the humerus and sternum and associated structures. Following retraction of the needle by a few millimeters, 0.2 ml of the solution was injected. The orientation of the needle was changed 3–4 times during the injection to enable all branches of the plexus to be reached. Assessment was made of motor and sensory blockade.

Thut et al. (1995) used the **rabbit** tooth-pulp assay (see H.1.2.8) to quantify efficacy and duration of antinociception by local anesthetics infiltrated into maxillary tissues.

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G.1.1.3 Conduction anesthesia on the mouse tail

PURPOSE AND RATIONALE

The radiant heat method as being used for evaluation of systemic analgesic activity (D'Armour et al. 1941; Ther et al. 1963; Grant et al. 1993) can also be used for determination of conduction anesthesia by injecting the local anesthetic into the root of the tail.

PROCEDURE

Groups of 10 mice (NMRI-strain) of both sexes with a weight between 18 and 22 g are used for each dose. Before administration of the test compound or the standard the normal reaction time is determined. The animal is placed into a small cage with an opening for the tail at the rear wall. The tail is hold gently by the investigator. By opening of a shutter, a light beam exerting radiant heat is directed to the proximal third of the tail. After about 6 s, the reaction of the animal is observed by the investigator. The mouse tries to pull the tail away and turns the head. The shutter is closed with a switch when the investigator notices this reaction. Mice with a reaction time of more than 6 s are not used in the test.

The test compounds and the standard are injected in a volume of 0.1 ml on both sides in the area of the tail root. The animals are submitted to the radiant heat again after 10 min. The area of heating is about 1.5 cm distal to the injection site. For each individual animal the reaction time is noted.

EVALUATION

There are two possibilities for evaluation:

1. The average values of reaction time after each time interval are calculated and compared with the pretest value by analysis of significance.
2. At each time interval only those animals which show a reaction time twice as high or higher as the pretest value are regarded as positive. Percentages of positive animals are counted for each time interval and each dose and ED_{50} values are calculated according to LITCHFIELD and WILCOXON.

MODIFICATIONS OF THE METHOD

The tail flick procedure using **rats** as test animals has been proposed by Herr et al. (1953).

Madan et al. (1970) determined conduction anesthesia by a tail-pinch technique in rats. The local anesthetic was injected subcutaneously bilaterally at the root of the tail. The test for local anesthesia was begun 15 min after injection by pinching with a polyethylene sheathed artery forceps and repeated every 15 min. The number of animals failing to remove the forceps by biting was recorded.

Grant et al. (1993) quantified the duration of the local anesthetic-induced conduction block in the mouse using the tail flick test.

Kamerling et al. (1985) described a method for studying cutaneous pain perception and analgesia in **horses**. This method was used by Harkins et al. (1996, 1997) for determination of highest no effect dose (HNED) for local anaesthetic responses to procaine, cocaine, bupivacaine, benzocaine and of a plant extract. A heat projection lamp was used as noxious stimulus directed onto the pastern to elicit a flexion-withdrawal reflex. Hoof withdrawal latency was defined as the time between lamp illumination and with-

drawal of the hoof. The local anesthetic drugs were injected into the area of the palmar nerve where it passes lateral to the sesamoid bone.

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G.1.1.4 Rabbit tooth pulp assay

PURPOSE AND RATIONALE

The rabbit tooth-pulp assay (see H.1.2.8) has been used successfully to evaluate the potency, efficacy, and duration of antinociception of analgesics (Yim et al. 1955; Piercey and Schroeder 1980; Wynn et al. 1984). Thut et al. (1995) described a method for administration of local anaesthetic drugs to the maxillary arch of rabbits and subsequent measurement of antinociceptive action.

PROCEDURE

Pulp chambers are exposed close to the facial gingival line of the two central incisors in New Zealand White male rabbits (1.5–2.0 kg) using a high-speed dental drill and 0.5-mm round burr immediately after the i.v. administration of 30 mg/kg ketamine. All experiments are conducted 48 h or more after pulp exposure. A linear ramp function stimulator is used as the direct current voltage source. The rabbit is slightly restrained, and the current is applied to the pulp via fine-wire platinum electrodes held in each of the cavities. Linearly rising voltage is applied at a rate of 0.33 V/s until a patterned lick-chew response occurs. Mean threshold voltages are established for controls using an average of 3 determinations, and then obtained after treatment using a single determination up to a maximum of 10 V. Rabbits having control values greater than 3 V are excluded from the study. The injection site of the test drug is 1.5 cm posterior to the central incisors and 4 mm below the roof of the maxillary buccal vestibule. The buccal vestibule is the area where the cheek and the top of the maxillary tissue join. The tip of a 27-Ga x 1/2" syringe needle is inserted until it contacts the cancellous bone. The volume of injection is 0.4 ml. Injections are made bilaterally so that the total volume injected is 0.8 ml. The animals can be used chronically, but no rabbit should be exposed to the same drug or the same dose more than once, and non should be injected more frequently than very third day. One animal is used per dosage when analgesia is observed to be either 0% or 100%. Two or 3 animals are used for dosages with effects between 0% and 100%. When more than one animal is used, data are averaged and treated as a single observation. Pulp chambers have to be re-exposed when the drilled openings disappear because of incisor growth (approximately after 3 weeks).

EVALUATION

The percentage of maximum effect (*MPE*) for each observation time is calculated from the following equation:

$$MPE = (TV - CV) / (10V - CV) \times 100$$

TV is the voltage after treatment, *CV* is the control voltage, and *10V* are the maximum volts applied. The voltage that elicits the response is recorded at zero time and 5, 10, 15, 30, 60, 120, and 180 min after injection. The *MPE* is calculated for each observation time. Graphs of the *MPE* values versus time are constructed, and the maximal effect and time of the maximal effect are recorded. Further, the duration of each dosage is defined as the length of time that that dose achieved a *MPE* greater than 25%. Data are regressed between observed time points to provide estimates of the time when 25% *MPE* is first achieved and also the time when 25% *MPE* is no longer observed. A computer program or the standard method of Litchfield and Wilcoxon (1949) can be used to calculate ED_{50}, ED_{90}, and ED_{95} values with 95% confidence limits.

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G.1.1.5 Retrobulbar block in dogs

PURPOSE AND RATIONALE
Defalque and Stoelting (1976) published a method to determine latency and duration of action of local anesthetics after retrobulbar injection in dogs.

PROCEDURE
Young female mongrel dogs weighing 13–15 kg are used. Twenty-four h before the test, 0.25% eserine ointment is placed in each conjunctival sac of the dog. Pentobarbital (25 mg/kg) is administered intravenously, then repeated with 10 mg/kg at hourly intervals, thus maintaining the animal in light anesthesia (corneal reflex present). Ten min after induction, the dog is put into 30-degree head-down position, and 20 ml of 0.05% tetracaine is forced into the epidural space through the interarcuate ligament. Horner's syndrome occurs within 5 min.

A 150-watt surgical lamp is now focused upon the eye from an 1 meter distance. Fifteen min later, a retrobulbar block is performed: The sclera is seized with an ophthalmic forceps and the eyeball is pulled downward and medially; a 23-gauge needle is then introduced through the superior rectus muscle, tangentially to the globe, and is immobilized as soon as a click indicates penetration of the retrobulbar space; correct placement is confirmed by free motion of the needle tip and protrusion-rotation of the eyeball upon injection of 1 ml of air.

After aspiration, 2 ml of the tested anesthetic is then injected at a rate of 0.5 ml per second. The pupil dilates and reaches its maximal diameter (6 mm) within a few minutes. This apparent diameter is estimated with a 2-cm long ruler calibrated in millimeters, whose center is gently applied to the corneal center. The pupil is measured every 15 s for 5 min, then every 5 min until reappearance of maximal miosis (pinpoint and asymmetrical), a precise endpoint which generally coincides with corneal reflex and lacrimation.

EVALUATION
Drug latency (in min) and duration (in 5-min units) are averaged for both eyes of each animal, and the mean and standard deviation then calculated for all test animals. Analysis of variance is performed to find significant differences between various local anesthetics.

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G.1.1.6 Isolated sciatic nerve preparation of the frog

PURPOSE AND RATIONALE
Isolated nerves are immersed between stimulating and recording electrodes in solutions containing local anesthetics which allows electrophysiological measurements (Paterson and Hamilton 1970).

PROCEDURE
A large frog is sacrificed by decapitation with a pair of scissors, and the spinal cord severed. The skin around the entire body in the region immediately posterior to the forelimbs is cut, and the abdomen and hind legs are skinned by peeling the skin in a posterior direction. The fat, muscles, and other tissues are cut at the posterior end of the body so as to expose the nerves extending posteriorly from the cord. The muscles of the thigh are cut and retracted so as to expose the sciatic nerve. Finally, the gastrocnemius muscle is removed and the peroneus muscle is extended laterally from the tibiofibula. The entire sciatic nerve with branches should now be exposed. A thread with a loop next to the nerve is tied around the nerve in the region of the sacral vertebra. The nerve is then cut anterior to the thread. During the isolation procedure the nerve is kept moist with frog Ringer solution. The connective tissue and the nerve branches are cut carefully. The nerve is finally cut near the distal end of the tibiofibula and extended on a filter paper previously soaked with Ringer solution. With the nerve anchored by the thread, the remaining connective tissue is peeled back toward the distal end of the nerve. Any remaining branches are cut at the point of their bifurcation. The distal end of the nerve is tied with a length of thread. The sciatic-peroneal nerve preparation is mounted in a nerve chamber for recording.

Electrodes for stimulation are placed on the proximal end of the nerve while recording electrodes are placed at the distal end. The nerve is immersed in a trough containing frog Ringer solution as bathing fluid. The stimuli are applied with a commercial stimulator (e.g., Grass Model S4) with a duration of 5 ms, a frequency between 30/s, and 1.5 to 15 mV. The recording electrodes are connected with an amplification and display system. Stimulation of the nerve produces a display on the oscilloscope. Adjusting the voltage setting on the stimulator will cause the action potential to increase and decrease correspondingly on the scope. After pinching the nerve between the last two recording sites, the action potential should be monophasic and appear entirely above the base line. The spike is observed periodically for several min to ascertain its stability.

The bathing trough is changed to a trough containing the local anesthetic dissolved in Ringer solution. At one or 2 min intervals the amplitude of the spike is read and recorded on a polygraph. After 5 min treatment, the drug trough is removed and the nerve is immersed in the Ringer solution of the bathing trough. Amplitudes are measured in periodic intervals and thus the recovery process is recorded.

EVALUATION

Block and recovery curves are achieved with various concentrations of the test compound and the standard. Dose-response curves can be obtained.

MODIFICATIONS OF THE METHOD

Specialized techniques with isolated nerves (desheathed nerves, equilibrium blocks, single nerve fibres) have been described by Camougis and Takman (1971).

Den Hertog (1974) used the **desheathed cervical vagus of the rabbit** mounted in a single sucrose gap apparatus and measured changes in the membrane potential and compound action potential. The ratio of the distance between stimulation and recording electrode (15 mm) and the time between stimulation of the nerve and the top of the action potential was taken as an index for the mean conduction velocity.

Salako et al. (1976) tested a new anti-arrhythmic drug as a local anesthetic on desheathed **frog** nerve.

Lee-Son et al. (1992) used the sucrose-gap method on desheathed isolated frog peripheral nerves to study the stereoselective inhibition of neuronal sodium channels by local anesthetics.

Lambert et al. (1994) studied the reversibility of conduction blockade in desheathed bullfrog sciatic nerves, using the sucrose gap method for recording compound action potentials, before and during exposure to local anesthetics and during drug washout.

Isolated nerves from other species have been used for the study of local anesthetics, such as those from rabbits by Ritchie et al. (1965a,b), or from rats by Condouris and Lagomarsino (1966), Štolc and Mai (1993).

Gissen et al. (1980) observed differential sensitivities of mammalian nerve fibers to local anesthetic agents in rabbits. A fibers were blocked at the lowest drug concentrations, the intermediate B fibers were blocked at a higher drug concentration, and the smallest, slowest-conducting C fibers required the highest drug concentration for conduction blockade.

Fink and Cairns (1984) showed differential slowing and block of conduction by lidocaine in individual afferent myelinated and unmyelinated axons in rabbits.

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G.1.1.7 Isolated mammalian sciatic nerve preparation

PURPOSE AND RATIONALE

Karlsson et al. (1994) tested the localanesthetic-like effects of an NK_1 receptor antagonist in a mammalian sciatic nerve preparation using guinea pigs.

PROCEDURE

Male guinea pigs weighing 250–400 are sacrificed and the sciatic-tibial nerves immediately excised and cleaned in buffer equilibrated with 95% O_2 + 5% CO_2 under a dissecting microscope. The nerve is mounted in a Plexiglas chamber consisting of three connecting wells. The central well is perfused with buffer at a rate of 5 ml/min and contained the grounding electrode (Ag/AgCl), the in- and outlet of the peristaltic pump driven perfusion system and a thermistor probe. The two lateral wells (one for stimulating, the other for recording) are filled with mineral oil. Stimulating and recording electrodes are made from bare platinum-iridium wire. The passages between the central and lateral wells are sealed with petroleum jelly. The length of the nerve exposed to the drug in the central well is 8 mm. The nerve chamber is kept at a temperature of 27.0 ±0.5 °C.

A constant current supramaximal stimulus of 50 µs duration is used throughout the experiment. The intensity of the stimulus is ten times greater than the threshold intensity required to elicit a compound action potential. Throughout the experiment the nerve is stimulated at the low frequency of 1/min to determine basal compound action potential amplitude. Once the response has stabilized, a single burst of pulses (pulse train duration 250 ms, at 40 Hz) is applied approximately 5 min before drug application (control train) and again after drug application to determine the extent of frequency dependent block. The compound action potentials are digitized with a computer interface and a software program and recorded on a PC. A two channel storage oscilloscope is used to follow the experiment and to automatically sample and calculate the amplitudes of the compound action potentials.

The drugs are dissolved in buffer and applied approximately 5 min after the control train. The development of basal block following drug application is followed with 0.0167 Hz stimulation. In the case of lidocain, the maximal inhibition is reached 20–30 min after application. The basal block is allowed to reach equilibrium, which is defined as the condition in which the difference in amplitude between the first and the last of five consecutive responses is less than 1% of the control compound action potential amplitude obtained prior to drug application. To assess frequency dependent block, a second stimulus is given after equilibrium is reached. Once basal and frequency dependent block has been determined, the nerves are washed with fresh buffer.

EVALUATION

The magnitude of the basal block (%) is calculated by comparing the average peak amplitude of the last three compound action potentials with the average peak amplitude of the last three compound action potentials prior to drug application. Similarly, frequency dependent block is expressed in percent of the control train, and is effectively the basal block plus the additional decrease in compound action potential amplitude. Dose-response curves for the standard and the test drugs are constructed by plotting basal block and frequency dependent block against drug concentration. The pIC_{50} values (the negative logarithm of the molar concentration of the drug producing 50% inhibition of the compound action potential amplitude) are estimated by linear regression analysis of the results in the 10–90% interval of the dose-response curves.

The dose-response data obtained with the standard and the test drugs and the effect of a single concentration (0.5 mM) of all compounds on basal block and frequency dependent block are analyzed by ANOVA. The values of latency to the peak of compound action potential are analyzed by paired t-test.

MODIFICATIONS OF THE METHOD

Fink and Cairns (1984) studied differential slowing and block of conduction by lidocaine in individual afferent myelinated and unmyelinated axons.

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G.1.1.8 Effect of local anesthetics on different nerve fibres

PURPOSE AND RATIONALE

Wildsmith et al. (1989) examined the *in vitro* sensitivities of different types of fibres in rabbit vagus nerves to local anaesthetic block with a range of local anaesthetic drugs at high and low frequency stimulation. Rapidly conducting, myelinated (A) fibres are more sensitive than more slowly conducting, unmyelinated (C) fibres.

PROCEDURE

The cervical portions of the vagus nerves of rabbits weighing 2.5 kg are removed, desheathed and mounted in an airtight chamber which is maintained at 37 °C by a water jacket. A central 1-cm section of the nerve chamber is perfused at 0.5 ml/min with carbonated Liley solution (pH 7.4 ±0.02 after equilibration with 5% carbon dioxide in oxygen). A tap system allows the perfusate to be changed to test solution without admission of air.

A square wave generator is used to apply supramaximal electrical stimuli (10–15 V for 1 ms) at a rate of 0.0167 Hz during a 30-min period of stabilization. Two trains of stimuli (duration 0.25 s) are applied, one at 20 Hz and the other at 40 Hz. The preparation is considered valid only if there is less than 5% decrement in height of the action potential during stimulation at 40 Hz. The signals from the nerve are amplified, digitized (Unilab 532.001 interface sampling at 40 µs intervals for 8 ms, then at 200 µs intervals for 50 ms) and recorded with a microcomputer based system. The numeric derivative of the signal can be printed out immediately or stored on disc for subsequent processing. Signals are monitored also on a storage oscilloscope.

A submaximal blocking concentration of the hydrochloride salt of a test drug dissolved in carbonated Liley solution is applied. Only one concentration of one drug is applied to each nerve. The stimulation rate remains at 0.0167 Hz until any changes in the compound action potential are complete (minimum period 30 min), when the trains of high frequency stimulation are repeated. Drug effect is measured as the percent decreases in the height of the three components (A, B and C fibres) of the compound action at each frequency of stimulation. At 20 and 40 Hz, the last signal produced by each train of stimulation is used for analysis. An experiment is considered valid only if the action potentials recover to more than 90% of control height on washing the drug from the nerve.

EVALUATION

From the data obtained, the ED_{50} (and its SE) for the effect of each drug on each fibre type at each rate of stimulation is determined by log-probit analysis. Plots of the development of block while the nerves are being stimulated at 0.0167 Hz are also made. From these an index of the rate of development of A and C fibre block is derived ($T_{1/2B}$). This is defined as the time taken to develop 50% of the eventual maximum degree of block for the particular fibre type in each experi-ment.

MODIFICATIONS OF THE METHOD

Scurlock et al. (1975) examined the relative sensitivity of sympathetic preganglionic and postganglionic axons, B and C fibres, respectively, to structurally dissimilar local anaesthetics. Cervical sympathetic trunks from adult rabbits were submerged in Krebs-Henseleit solution except during the brief recording periods, when they were stimulated electrically with twice maximal square-wave pulses of 0.15 ms (B fibres) or 0.5 ms (C fibres) duration. After control records, the bath chambers were filled with various concentrations of local anaesthetics and the action potentials recorded at 5-min intervals. The decrease of action potentials of the B- and C-fibres was recorded separately.

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G.1.1.9 Measurement of sodium and potassium conductance in voltage clamp experiments

PURPOSE AND RATIONALE

Voltage clamp experiments on single nodes of Ranvier can be performed with single myelinated nerve fibers from the sciatic nerve of the frog (Stämpfli 1954, 1968; Nonner 1969; Nonner et al. 1975; Stämpfli and Hille 1976; Borchard and Drouin 1978). Sodium, potassium and leakage currents (I_{Na}, I_K, I_L) can be measured.

Descriptions of structure and function of voltage-gated sodium channels were given by Catterall (1988, 1992), Stühmer (1991), Narahashi and Herman (1992), Cohen and Barchi (1993).

PROCEDURE

Single myelinated nerve fibers are dissected from the sciatic nerve of the frog (*Rana esculenta*). The fibers are bathed in a Ringer solution containing 110 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 5.0 mM Tris, pH 7.3, at 15 °C. For measurement of sodium, potassium and leakage currents (I_{Na}, I_K, I_L), the nodal membrane is clamped to a holding potential of –30 mV (relative to the resting potential of –70 mV). Hyperpolarizing prepulses (–40 mV, 50 ms) followed by 2 ms test pulses (60 mV) are applied every 5 s to record peak sodium currents, I_{Na}, and to avoid the influence of accumulation of frequency-dependent block. Every second pulse is followed by an afterpulse (120 mV, 20 ms) to measure steady state I_K. The leakage current, I_L, is measured at the end of the hyperpolarizing prepulse and recorded every 10 s.

After superfusion of the nodal membrane with a local anesthetic, a quick and then a slow decrease of the peak sodium current (I_{Na}) occurs. When the application of the local anesthetic is stopped, I_{Na} increases quickly and then slowly within min indicating a reversible action of the local anesthetic on the nerve fiber. With local anesthetics, the potassium current (I_K) behaves similarly, whereas the leakage current (I_L) remains unchanged.

Dose dependent action of local anesthetics on I_{Na}-V relations

The holding potential is set at –30 mV. A hyperpolarizing prepulse (–40 mV, 50 ms) is followed by a depolarizing test pulse (30 ms) with varying amplitudes. Local anesthetics induce a dose-dependent reduction of sodium-inward and -outward currents over the whole range of voltage clamp steps. The measurements are performed at extracellular pH 7.3.

Dose dependent action of local anesthetics on I_K-V relations

Potassium currents, I_K, are measured at the end of depolarizing test pulses of 30 ms duration. Local anesthetics induce a dose-dependent decrease of potassium currents at extracellular pH 7.3.

EVALUATION

The effects on sodium, potassium and leakage currents (I_{Na}, I_K, I_L), the dose dependence of action on I_{Na}-V relations, and the dose dependence of action on I_K-V relations of various local anesthetics are compared.

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G.1.2 Infiltration anesthesia

G.1.2.1 Infiltration anesthesia in guinea pig's wheals

PURPOSE AND RATIONALE

Based on earlier work by McIntyre and Sievers (1937) and Sievers and McIntyre AR (1938), the use of intracutaneous wheals in guinea pigs was recommended by Bülbring and Wajda (1945). This method has become a standard operating procedure for testing local anesthetics.

PROCEDURE

Adult guinea pigs of either sex weighing 250–300 g are chosen. On the day preceding the experiment the hair on the back is clipped and two areas of 4–5 cm diameter are shaved. This produces a certain amount of irritation which disappears overnight. The sensitivity of the skin is greatest in the midline and slightly more so in the front than in the back area. For this reason each concentration of a local anesthetic must be tested in both areas. Six tests using three guinea pigs can be performed simultaneously. The doses of local anesthetics are always injected intracutaneously in 0.1 ml saline. Three guinea pigs receive one dose in the front area and another dose in the back area; the size of the wheal is marked with ink. One side is used for the test preparation, the other side for the standard (e.g., 1% butanilicaine). The reaction to pin prick is tested 5 min after injection in the following way. After observing the animal's normal reaction to a prick applied outside the wheal, six pricks are applied inside the wheal and the number of pricks is counted to which the guinea pig fails to react. The pricks are applied at intervals of about 3–5 s. Six pricks are applied every 5 min for 30 min. Having completed the test on 3 guinea pigs, the same solutions are injected into 3 other guinea pigs, but the solution which was used for the front is now used for the back area and vice versa.

EVALUATION

The number of times the prick fails to elicit a response during the 30 min period is added up, and the sum, out of possible 36, gives an indication of the degree of anesthesia. Using various doses, dose-response curves can be established. For time-response curves, the prick tests are repeated every 10 min. Half-life times are calculated as the time, when after complete anesthesia 3 out of 6 pricks elicit again a response (Ther 1953a,b).

MODIFICATIONS OF THE METHOD

The test can also be used to study the influence of vasoconstrictors, such as adrenaline.

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G.1.3 Surface anesthesia

G.1.3.1 Surface anesthesia on the cornea of rabbits

PURPOSE AND RATIONALE

Following the pioneering work of Sollmann (1918), block of the rabbit corneal reflex as described by Régnier (1923) has become a standard test method for evaluating local anesthetics (Quevauviller 1971).

PROCEDURE

Albino rabbits of either sex weighing 2.5–3 kg are placed into rabbit holding cages. The upper and lower eyelashes are carefully clipped. The conjunctival sac

of one eye is held open, thus forming a pocket. From a 1 ml syringe with a 22-gauge needle, 0.5 ml of a solution of the anesthetic is applied into the conjunctival sac for 30 s. Then the procedure is repeated, so that 1.0 ml is applied within 1 min. One ml of the standard (0.1% solution of tetracaine hydrochloride) is applied to the other eye. Effective local anesthetics extinguish the corneal reflex (blinking) elicited by any touch of the cornea. For quantitative purposes, the irritation with a bristle according to von Frey (1894, 1896, 1922) has been recommended. An equine hair bending at a load of 230 mg is attached perpendicularly to a glass rod. Within 25 s, the cornea is touched 100 times. The summation of many stimuli applied this way gives better results than a single touch with a glass rod (Ther and Mügge 1953). The test is started 5 min after application of the drug and repeated every 5 min until anesthesia vanishes and blinking occurs again. The time between disappearance and reappearance of the corneal reflex is registered.

EVALUATION

Using the time of loss of the corneal reflex as parameter after application of different doses, dose-response curves can be established and potency ratios versus the standard calculated.

MODIFICATIONS OF THE METHOD

Chance and Lobstein (1944) used **guinea pigs** for testing surface anesthesia by the corneal reflex.

Bartsch and Knopf (1970) described an electrically operated stimulator with a bristle allowing variable frequencies to evaluate surface anesthesia on rabbit cornea.

Hotovy (1956) investigated the synergism between the local anaesthetic compound dibucaine hydrochloride applied to the conjunctival sac of rabbits and intravenously administered analgesics using Régnier's method

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G.1.3.2 Suppression of sneezing reflex in rabbits

PURPOSE AND RATIONALE

Nieschultz et al. (1958), Åström und Persson (1960) used the sneezing reflex in rabbits to test local anesthetic activity.

PROCEDURE

Groups of male rabbits weighing 3 kg are used. Using a cotton tampon, the test solution is applied to the mu-

cous membrane of one nostril. The solution of a standard local anesthetic is administered to the nasal mucosa of the other nostril. After 2 min the mucous membrane is stimulated by a fine pencil. Loss of the sneezing reflex is regarded as sign of complete anesthesia. The stimulation is repeated after 3, 6, 10 and 15 min and continued every 5 min until the sneezing reflex reappears. Various concentrations of test compound and standard are applied.

EVALUATION

Using the loss of the sneezing reflex as parameter after application of different doses, dose-response curves can be established and potency ratios versus the standard calculated. Furthermore, the duration of activity can be evaluated.

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G.1.4 Epidural anesthesia

G.1.4.1 Epidural anesthesia in guinea pigs

PURPOSE AND RATIONALE

Activity and tolerability of new local anesthetics after epidural injection have to be studied in various animal species in order to predict both parameters in patients. A method to test epidural anesthesia in guinea pigs was described by Åkerman et al. (1988).

PROCEDURE

Male guinea pigs weighing 300–500 g are anesthetized by means of an intraperitoneal injection of an aqueous solution of chloral hydrate 42.5 g/l; ethanol 90 g/l; propylene glycol 428 g/l; sodium pentobarbitone 9.75 g/l; and magnesium chloride 21 g/l. A skin incision is made from the level of the lumbosacral fossa and approximately 1.5 cm down in order to expose the sacral area in the mid-line. With the vertebral column flexed, the lumbosacral intervertebral ligament is carefully incised. Through this small opening a polyethylene catheter (PE 10) is inserted maximally 1.5 cm along the roof of the vertebral canal to the L4–L5 region. The catheter is sutured to the overlying lumbar fascia which is then closed. The catheter is tunneled under the skin and exteriorized through an incision in the neck region. After fixation of the catheter to the fascia of the neck muscles and suturations of the incisions, the catheter is filled with saline and sealed.

After a recovery period of at least 1 day, 0.1 ml of 2.0% lidocaine is injected over a period of 1 min, and the motor and sensory blocks are assessed. The injection of lidocaine which results in a bilateral, reversible blockade indicates a successful preparation. A minimum of 8 animals are used in the further experiments for each test solution.

EVALUATION

Mean time to onset of block and mean duration of block are calculated from number of legs blocked.

MODIFICATIONS OF THE METHOD

Siems and Soehring (1952) described a model in **guinea pigs** resembling peridural and paravertebal anesthesia in man.

A technique for epidural administration in the **dog** was described by Feldman and Covino (1988).

Defalque and Stoelting (1966) used a standard veterinary epidural technique to study latency and duration of action of some local anesthetic mixtures in **dogs**. The animals, prone and with their spread-out extremities attached to the table, were placed in 40-degree Trendelenburg position. Under sterile conditions, the epidural space was penetrated through the interarcual ligament with a short-beveled No. 22 spinal needle. After identification of the epidural space (aspiration, air injection), 4 ml of the investigated solution were injected with a constant injection rate of 2 ml per sec. Absence to skin-twitch response to pinching with an Allis clamp was considered analgesia. This was tested on both flanks, along a line 2 cm off the spine. Disappearance of contraction of the anal sphincter in response to stroking all quadrants of the anal margin closely correspond to complete analgesia at the level of the interarcual ligament; since this was an easily measurable parameter, disappearance and recurrence of this reflex was chosen as endpoint of latency and duration of action, respectively.

Chernyakova et al. (1994) studied the effects of azacaine during epidural anesthesia in **rabbits**.

Hughes et al. (1993) described a rabbit model for the evaluation of epidurally administered local anaesthetics. A "loss of resistance technique" similar to that employed in caudal epidural injection in humans was used. The rabbit was carefully restrained by an assistant. The readily palpable cranial dorso-iliac spines, lying on either side of the prominent spinous process of the seventh lumbar vertebra, served as landmarks. The thumb and the middle finger of the left hand were

placed on the two crests and the left index finger used to palpate the midline, L7 spine and the depression over the lumbosacral fossa. With the index finger in position on the L7 spine to serve as a guide, a short bevel 1.5 cm 20 gauge spinal needle was introduced a right angles to the skin in the midline with the bevel aligned longitudinally. After passage through the skin, only minor resistance was felt until the ligamentum flavum was reached. When passing through the ligament, a definite "pop" was felt and resistance to advancement of the needle was lost. When correctly placed, the needle was at a depth of approximately 0.75 to 1.0 cm and firmly held by the ligament. The stylet was then withdrawn and the hub inspected for the presence of blood or cerebrospinal fluid. If absent, the needle was rotated through 90° to direct the bevel caudally, a 1.0 ml syringe was attached and 0.1 ml of air injected. Accurate placement was indicated by the absence of resistance to injection and lack of subcutaneous crepitus. A syringe containing the desired dose of the local anesthetic was attached and the solution injected over a 5–10 s period. The pharmacodynamic responses were assessed by (1) sensory loss, (2) loss of weight-bearing ability, and (3) flaccid paresis.

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G.1.5 Intrathecal (spinal) anesthesia

G.1.5.1 Spinal anesthesia in rats

PURPOSE AND RATIONALE

Activity and tolerability of new local anesthetics after intrathecal injection have to be studied in various animal species in order to predict both parameters for spinal (subarachnoid) anesthesia in patients. Intrathecal injections to rats or mice are performed according to the method of Hylden and Wilcox (1980) or Åkerman (1985) as being used by Ossipov et al. (1988).

PROCEDURE

Male Sprague-Dawley rats weighing 50–75 g are used. The rat is held firmly by the pelvic girdle. A 30-gauge needle is attached to a 25-µl Hamilton syringe is inserted into the tissue on one side of the L5 or L6 spinous process at an angle of about 20°. The needle is advanced to the groove between the spinous and transverse processes and then moved forward the intervertebral space at an angle of about 10°. About 0.5 cm of the needle is then in the vertebral column. Correct placement of the needle is indicated by an arching of the tail. Drugs are dissolved in saline or water and administered in a volume of 5 µl.

Antinociception is determined in a modification of tail flick assay in rats by placing the tail of the rat under a focused radiant heat source.

EVALUATION

The degree of antinociception is defined as the percentage of maximum possible effect. This percentage is determined for each dose at each time measured allowing to calculate ED_{50} values.

MODIFICATIONS OF THE METHOD

Omote et al. (1995) studied the effects of verapamil on spinal anesthesia induced by local anesthetics administered via a chronic intrathecal polyethylene catheter in **rats**. The catheter was inserted 15 mm cephalad into the lumbar subarachnoid space at the L4-5 vertebrae with the tip located near the lumbar enlargement of the spinal cord. The catheter was tunneled subcutaneously and externalized through the skin in the neck region. At least 6 days of postsurgical recovery was allowed before animals were used in experiments. The tail-flick and the mechanical paw pressure tests were used to assess thermal and mechanical nociceptive thresholds.

Mestre et al. (1994) described a method for performing direct intrathecal injections in **rats** without introducing a spinal catheter.

Yaksh and Rudy (1976) described a procedure of chronic catheterization of the spinal subarachnoid space in **rats** and **rabbits**. A polyethylene catheter (PE-10) was inserted through a puncture of the atlanto-occipital membrane into the spinal channel in anesthesia and secured to the skull. In this way, drugs could be administrated into the spinal subarachnoidal space of unanesthetized animals.

Cole et al. (1990) used this model to determine the influence of spinal tetracaine on central nervous system metabolism during nociceptive stimulation in **rats**.

Bieter et al. (1936) described a method of inducing spinal anesthesia in the **rabbit** and determined threshold anesthetic and lethal concentrations. Injections were performed at the lumbo-sacral union between the spinous process of the last lumbar vertebra and the first sacral spinous process. A dose of 0.02 ml per centimeter of spinal length was chosen as standard volume for determining minimal anesthetic and minimal lethal doses.

Langerman et al. (1991) studied the duration of anesthesia after a single subarachnoid injection of a local anesthetic in **rabbits**. Tetracaine 1% 0.5 mg/kg was administered in 10% glucose or in lipid solution via catheters chronically implanted in the subarachnoid space. The pharmacologic effect was assessed by evaluating the intensity and duration of motor blockade according to a three-stage scale.

Bahar et al. (1984) performed chronic implantations of nylon catheters into the subarachnoid space of Wistar **rats** and **marmosets** and tested the effects of local anesthetics.

Åkerman et al. (1988a,b) used **mice** to study spinal morphine antinociception potentiation by local anesthetics.

A simple technique for intrathecal injections by lumbar puncture in unanesthetized **mice** was described by Hylden and Wilcox (1980).

This model has been used by Langerman et al. (1994) to evaluate the potency of various local anesthetics. Adult Swiss Webster male mice were slightly anesthetized with halothane and the skin overlying the dorsal lumbar spine was opened using a transverse incision 8–10 mm. Animals were allowed to recover for 1 h before the evaluation of the baseline nociceptive response latency. Thirty min after baseline testing, intrathecal local injections were performed with an automatic syringe fitted with a 30-gauge needle. Fourth spinal space was identified and the needle was inserted between the two spinal processes in a cephalad direction at a 20° angle. Resistance encountered at approximately 5–6 mm from the skin indicated proper location of the needle in the spinal canal. A fixed 10 µl volume of injectate was used. Analgesia was measured using the tail-flick test based on tail withdrawal in response to heat generated gy a light beam focused on the ventral tail surface.

A chronic model for investigation of experimental spinal anesthesia in the **dog** was described by Feldman and Covino (1981).

Dohi et al. (1987) inserted a polyethylene catheter into the lumbar subarachnoid space in **dogs** through a small hole in the dura for administration of drugs. The tip of the catheter was placed approximately 2–3 cm cephalad to the lumbar electrode introduced for recording of hydrogen clearance to measure spinal cord blood flow.

Kozody et al. (1985) measured spinal cord and spinal dural blood flow in the cervical, thoracic and lumbosacral regions in **dogs** using the microsphere technique. Measurements were taken 20 and 40 min after lumbar subarachnoidal injection through the L5-L6 or L6-L7 interspace.

The technique of evaluation of spinal anesthesia by a local anesthetic in the **Rhesus monkey** was described by Denson et al. (1981).

Kyles et al. (1992) described a simple and noninvasive method for the chronic implantation of intrathecal catheters in the **sheep**.

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G.1.5.2 Blockade of urethral reflex in rabbits

PURPOSE AND RATIONALE

For evaluation of spinal anesthetics the test substance has to be injected into the spine. For the study of local anesthetics for which conduction anesthesia has been verified, intraspinal injection provides more complete information. Spinal anesthesia has been studied in rabbits (Bieter et al. 1936; Luduena and Hoppe 1951; Luduena 1957; Gonzalez and Luduena 1961; Turner RA 1965), cats (Sechzer 1965), and dogs (Dvorak and Manson 1930). If the solution of test substance is injected intrathecally to male rabbits, the presence of anesthesia may be determined by the urethral reflex (Luduena 1957).

PROCEDURE

Male Chinchilla rabbits weighing 3.0–3.5 kg are used. The volume administered intrathecally is 0.02 ml per centimeter of spinal length. It is injected at a rate of 1 ml per min with a 22-gauge needle that is 3.8 cm long. The needle is introduced between the sixth and seventh lumbar vertebrae, and not through the lumbosacral space. The needle, held as lightly as possible between the thumb and forefinger, is introduced slowly until a typical sharp and sudden twitch occurs. This indicates penetration into the subarachnoidal space. A one ml tuberculin syringe is then attached to the needle. It is not possible to aspirate fluid after insertion of the needle. However, it is possible to aspirate a portion of the injected solution. Very little pressure is required for injection of 0.4 to 0.6 ml of anesthetic solution while the animal is restrained in a canvas hammock. A catheter is inserted into the urethra, where it is kept without digital pressure. At 15- or 20-s intervals, 2 to 3 ml of water, at room temperature, are injected rapidly into the catheter. This causes the "urethral reflex", consisting of retraction of the penis and contraction of the anal sphincter. The water runs out of the urethra around the catheter. Without medium or high concentrations of the anesthetics the reflex is absent on the first reading. After loss of the urethral reflex, the test is repeated every minute or two for 15 min, and thereafter at longer intervals. The duration of urethral areflexia is taken as the time of the first positive reading (reflex absent) to the middle of the interval between the last positive and the first negative reading. This duration varies linearly with the logarithm of concentration.

EVALUATION

The threshold anesthetic concentration in grams per 100 ml for abolition of the urethral reflex for 5 min, the TAC_5, is a standard for comparison. There is a correlation between activity and systemic toxicity. The ratio of the activity to the irritancy is the most important parameter.

MODIFICATIONS OF THE METHOD

Burdyga and Magura (1986) studied the effects of local anesthetics on the electrical and mechanical activity of the guinea-pig ureter.

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G.1.6 Endoanesthetic effect

PURPOSE AND RATIONALE

Endoanesthesia has been described as the effect of local anesthetics on viscero-afferent receptors, located predominantly in the lung as distension receptors (Meier and Bein 1950; Zipf 1953, 1957, 1959, 1966. 1968; Zipf et al. 1955; Zipf and Oehler 1955; Reichertz et al. 1957; Zipf and Reichertz 1957; Zipf et al. 1963; Wellhöner and Conrad 1965; Siemoneit et al. 1966; Dittmann and Zipf 1973; Borchard 1979). The endoanesthetic effect has been claimed to be the basis of an antitussive action of local anesthetics (Bucher 1956; Bein and Bucher 1957; Kraushaar et al. 1964).

PROCEDURE

Male guinea pigs weighing 300–400 g are anesthetized by intraperitoneal injection of 1.25 g/kg urethane. The studies are carried out on spontaneously breathing or artificially respirated monovagotomized or bivagotomized animals. Intravenous injections are given via a catheter into the jugular vein. The flow rate of the respiratory air is measured with a Fleisch tube connected to a pneumotachograph. In experiments with positive pressure respiration, the open end of the Fleisch tube is attached to a respiratory pump (40 strokes per min). A cannula is inserted into the carotid artery and connected to a pressure transducer.

The left nervus vagus is exposed from the thorax aperture until his entry into the foramen jugulare and cut in the middle of the distance. Both ends are placed over 3 wires of Ag/AgCl 6-way electrodes. The edges of the skin wound are pulled upwards with clamps and the space around the trachea is filled with paraffin to prevent the nerve from drying out. The afferent and efferent vagus potentials and also their integration curve (Zipf and Reichertz 1957) are recorded on a direct recorder. In addition, the ECG in the second lead is recorded continuously. The animal is shielded electrically with the aid of a Faraday cage.

The nervus vagus conducts not only afferences from the lung but also from other organs to the brain. The base points of the integral curve, which correspond to the vagus action potentials being synchronous with inspiration and issued by the pulmonary distension receptors, therefore lie above the baseline of the recorder tracing. The vagus afferences which remain after subtraction of the vagus activity synchronous with respiration ("inspiratorial activity") are called "residual activity". The level of inspiratorial activity depends both on the bronchial width (degree of inflation of the lungs) and on the functional state of the pulmonary receptors and of the afferent pathway. The occurrence of a bronchospasm is detectable from the decrease in the pneumotachogram, if the respiratory rate is not reduced at the same time.

EVALUATION

The following parameters are evaluated:

Afferent and efferent inspiratorial vagal activity, maximum flow rate of the respiratory air during inspiration and expiration, tidal volume, duration of the respiratory phases (inspiration = t_i, expiration = t_e, respiratory pause = t_p), heart rate and blood pressure.

The decrease of electrical activity after various doses of standard and test drugs is calculated at different time intervals after drug administration. Maximum of activity and decay with time are registered.

Statistical analysis is performed using the paired and non-paired *t*-test, after the applicability has been first checked with the F-test.

CRITICAL ASSESSMENT OF THE METHOD

The correlation between antitussive and local anesthetic activity has been challenged by Sell et al. (1958) and by Ther and Lindner(1961).

MODIFICATIONS OF THE METHOD

Bein and Bucher (1967), Wellhöner and Conrad (1955) tested various compounds for their anesthetic effect on pulmonary stretch receptors of **rabbits**.

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G.1.7 Effect on electroretinogram

PURPOSE AND RATIONALE

The retina, which belongs in ontogenesis to the central nervous system, can be prepared without damaging the parenchyma. The transretinal potential which can be induced by a light flash can be used as an objective criterion for the functional state (Sickel et al. 1960). The multi-phase potential path of the electroretinogram consists of an initial a-wave, a subsequent b-wave and an e-wave which starts with a delay after the end of the stimulus. While the amplitudes of the a- and e-wave are in the order of 50 μV, the amplitude of the b-wave (ϕ_b) is 500–1 000 μV. The electroretinogram can be used for various purposes, such as the evaluation of local anesthetics (Borchard 1979).

PROCEDURE

Frogs (*Rana esculenta*) are kept in dark for 1 h before beginning of the preparation. The frog is sacrificed by decapitation, the eyeball excised in dim red light and cut into two halves with the aid of a rotating blade. The retina together with the pigmented epithelium and choroid is removed from the rear half of the eye and transferred to a dish with Tyrode solution. The retina is then detached from the pigmented epithelium by careful shaking and spread out on a Monodur net. The net is fixed to a round carrier with a narrow ring and the chamber is transferred to a flow-through apparatus. An electrical pump is used for superfusion of the retina with Tyrode solution.

In order to apply the local anesthetic only to the receptor or vitreous body side of the retina, an apparatus is used in which the tissue on a silk net is stretched as the partition between two chamber halves which can perfused separately (Borchard and Erasmi 1974).

The electroretinogram is recorded with Ag/AgCl electrodes. After amplification, the signal is registered on a recorder or an oscillograph. Light stimuli of 10 ms are generated. The light from a low-voltage light source is focused on a electromagnetic shutter with a stimulus frequency of 0.1 Hz. Light stimuli of 1 s duration are used at intervals of 3 min to measure the influence of the local anesthetics on the electroretinogram as a function of light intensity.

All experiments are performed after adaptation to darkness and at room temperature. The amplitude of the b-waves (ϕ_b) which is measured from the lowest point of the a-wave to the maximum of the b-wave is used for evaluation. In order to obtain stationary test conditions, the experiment is started after a 45 min adaptation period. The stimulus signal is recorded by means of a photocell inserted in the light path.

Various concentrations of the local anesthetics are applied and various light intensities are used. The decrease of the b-wave starts immediately and then progresses slowly and continuously. After changing over to Tyrode solution, the decrease in the exposure potential is completely reversible.

EVALUATION

After various doses, time-response curves can be drawn and for a given time interval ED_{50} values can be calculated. Moreover, the influence of local anesthetics on the b-wave at various light intensities can be estimated.

MODIFICATIONS OF THE METHOD

Isolated retina for electroretinogram has been used not only from **frogs** but also from **rats** (Huang et al. 1991; Doly et al. 1993), **rabbits** (Mochizuki et al. 1992; Maynard et al. 1998) and from **beef** (Gosbell et al. 1996; Walter et al. 1999).

Moreover, the electroretinogram has been used as parameter for many *in vivo* studies with different purposes, e.g., on the effect of drugs after retinal ischemia, in various animal species, such as **rats** (Sugimoto et al. 1994; Hotta et al. 1997; Biró et al. 1998; Block and Schwarz 1998; Estrade et al. 1998; Ettaiche et al. 1999; Li et al. 1999), **rabbits** (Takei et al. 1993; Zemel et al. 1995; Horiguchi et al. 1998; Jarkman et al. 1998; Liang et al. 1998), **cats** (Imai et al. 1991; Ostwald et al. 1997; Kim et al. 1998), **dogs** (Jones et al. 1995; Yanase and Ogawa 1997) or **monkeys** (Tagliati et al. 1994).

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G.2 Neuromuscular blocking activity

G.2.0.1 General considerations

Neuromuscular transmission is mediated by mediated by nicotinic acetylcholine receptors for which various subtypes are described (Sargent 1993; McGehee and Role 1995; Karlin and Akabas 1995; Alexander et al. 2001).

Neuromuscular blocking agents are distinguished by whether or not they cause depolarization of the motor end plate. They are classified either as competitive (stabilizing) agents, of which d-tubocurarine is the classical example, or as depolarizing, desensitizing agents such as succinylcholine.

The **rabbit head-drop method** was described by Varney et al. (1948, 1949), Burn et al. (1952), Levis et al. (1953). Rabbits were given an interrupted intravenous injection at a rate of 0.1 ml every 15 s until the muscles supporting the head become sufficiently relaxed to prevent the head to be raised when the back is stimulated. This method has been replaced by the rabbit sciatic nerve-gastrocnemius muscle preparation, the isolated phrenic nerve diaphragm preparation of the rat, and the chick sciatic nerve-tibialis anticus muscle preparation.

The method described by Allmark and Bachinski (1949) used an inclined screen for testing curare-like activity in **rats**.

Skinner and Young (1947) placed **mice** weighing 15–17 g in sloping rotating cylinders. Mice falling away during 20 min after subcutaneous injection were considered as reactors. Dose response-curves and potency ratios could be calculated from the logarithmic dose-response curves using different doses of test compound and the standard tubocurarine chloride.

Collier et al. (1949) used a rotating drum to assess the activities of paralysant, convulsant and anaesthetic drugs in mice.

Fatt and Katz (1951) performed an extensive study on neuromuscular junction by recording end-plate potentials from curarized frog sartorius motor end-plates, using a KCl-filled microelectrode inserted into the muscle fiber in the region of the motor end-plate.

Electrophysiological analysis of transmission at the skeletal neuromuscular junction was reviewed by Prior et al. (1993).

A review on new neuromuscular blocking drugs was given by Hunter (1995).

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G.2.0.2 Isolated phrenic nerve diaphragm preparation of the rat

PURPOSE AND RATIONALE

The isolated phrenic nerve diaphragm preparation of the rat was originally described by Bülbring (1946) to study the influence of adrenaline on tissue functions normally elicited by acetylcholine. The method has been modified and is widely used by many investigators for studying drugs affecting the neuromuscular transmission.

PROCEDURE

Adult male Wistar rats are used. The animal is sacrificed and the blood is drained. The skin is removed from the middle of the chest. The thorax is opened and the front part of the left thoracic wall is removed. The phrenic nerve can bee seen quite distinctly. The nerve is cut just below the thymus and a thread is attached to the cut end. The nerve is than freed carefully from the attached tissue. However, no attempt is made to clean the nerve completely from all attached tissue. An incision is made in the left abdominal wall just below the diaphragm. Two converging cuts are made through the diaphragm and the ribs towards the tendinous part of the diaphragm with the phrenic nerve attached to the center of the diaphragm. The fan-like preparation is about 3 mm wide at the tendinous end and is about 15 mm wide at the costal margin. A thread is attached to the tendinous part of the diaphragm.

The preparation is fixed by a stainless steel rod with a pair of pins hooked to the rib. It is lowered into an organ bath and the thread from the muscle is attached to a force transducer. The nerve is stimulated with a pair of electrodes with a hole about 1 mm wide. The right phrenic nerve-diaphragm preparation is isolated in the same manner. The organ bath containing Tyrode solution with 2.0 g/l glucose is oxygenated at 37 °C with 95% O_2 and 5% CO_2. The nerve is stimulated 12 times per min by rectangular-wave pulses of 0.5 ms duration at 3–5 V. Contractions are isometrically recorded

through a transducer on a polygraph. The test drugs are left in the organ bath either for short periods of time (3–8 min) or for as long as the maximum effect can be observed. After a wash-out period of 3–5 min, the next dose can be added.

EVALUATION

The force of contractions after addition of various doses of the test drug is compared with the effect seen prior to drug application. Dose-response curves can be established.

CRITICAL ASSESSMENT OF THE METHOD

The isolated phrenic nerve diaphragm preparation of the rat is an excellent method for determining the potency of a drug to block or facilitate neuromuscular transmission. However, it is not a good preparation for differentiating between depolarizing and non-depolarizing neuromuscular blocking agents because, in many cases, depolarizing blocking agents fail to demonstrate initial facilitation and fail to reverse the effect of non-depolarizing blocking agents. For differentiating between depolarizing and non-depolarizing blocking agents the chick sciatic nerve-tibialis anticus muscle preparation is preferred. In this preparation, the curare-like drugs produce a neuromuscular blockade whereas the decamethonium-like drugs induce a contracture of the slow fiber when the neuromuscular transmission is blocked.

MODIFICATIONS OF THE METHOD

Colbert et al. (1990) studied the effects of temperature on the experimental reliability of the isolated rat phrenic nerve/diaphragm preparation.

Van Riezen (1968a,b) described the sciatic nerve-tibialis anticus muscle preparation in **chicks**. Three to 8 day old chicks are decapitated and the skin of the legs is rapidly removed. The leg is separated from the body by cutting through the hip joint. The muscles of the thigh are dissected and the sciatic nerve with the superficial peroneal branch is freed from the upper leg tissue. The fascia is removed from the lower leg and the tibialis anticus tendon identified. A thread is attached to the tendon of the muscle and the tibialis anticus muscle is freed towards but not up to the knee joint attachment where the nerve enters into this region. The upper and lower leg bones are then cut off leaving the muscle with its nerve attached to the knee joint. The tendon is fasted by a hook to a ring in the bottom of the organ bath. A thin steel rod is attached to the knee joint. The contractions of the muscle are recorded isometrically. The nerve is passed through an electrode similar to that used in the rat phrenic nerve-diaphragm method. The nerve is stimulated six times per min for 0.5 ms duration at supramaximal voltage.

Jenden et al. (1954) described the isolated lumbrical muscle of the **rabbit** as a preparation which is sensitive to competitive (*d*-tubocurarine-like) and depolarizing (decamethonium-like) blocking agents. This muscle is cylindrical in shape, about 18 mm long and 1 mm in diameter and has a wet weight of about 15 mg.

Jenden (1955) used the isolated diaphragm of **guinea pigs** to study the effect of drugs upon neuromuscular transmission.

Hoppe (1955) reviewed the potency of neuromuscular blocking agents in various species.

Birmingham and Hussain (1980) used the phrenic nerve-diaphragm and the hypogastric nerve-vas deferens preparation of the guinea pig for comparison of the skeletal neuromuscular and autonomic ganglion-blocking potencies of non-depolarizing relaxants.

Wessler and Kilbinger (1986), Wessler and Steinlein (1987), Wessler et al. (1992) described a modified **rat** phrenic nerve-hemidiaphragm preparation whereby most of the muscle was cut off (end-plate preparation).

Muir et al. (1989) used biventer cervicis nerve-muscle preparations from **young chickens** and phrenic nerve-hemidiaphragm preparations from **rats** to evaluate neuromuscular blocking agents. Micro-electrode recordings were obtained from the nerve-hemidiaphragm preparation of the rat and from the costocutaneous nerve-muscle preparation of the North American **garter snake** (*Thamnophis sirtalis*). The later preparation is particularly suitable for voltage clamp recording from the neuromuscular junction because it possesses large diameter fibres which aid visualization and penetration of endplates. This, coupled with compact endplates, allows good control of membrane voltage over the entire endplate region when using the two-microelectrode voltage clamp technique.

In order to record evoked endplate currents without accompanying muscle contraction, cut fibre preparations were used. Dissection and cutting of muscle fibres was performed at low K^+ (2 mmol/L) physiological solution perfused for approximately 30 min. Snake nerve-muscle preparations were mounted in a physiological salt solution of pH 7.1–7.2 containing (mmol/l) NaCl 159, KCl 4.2, $CaCl_2$ 1.5, $MgCl_2$ 4.2, HEPES 10. Rat nerve-muscle preparations were mounted in Krebs solution of the same composition as that used for tension experiments. The muscles were mounted in a Sylgard-coated Perspex dish and endplates were voltage clamped using glass capillary microelectrodes (resistance 2–10 MΩ). Voltage recording electrodes were filled with potassium chloride 3 mol/l and current passing electrodes were filled with potassium sulphate 0.6 mol/l.

The nerves were stimulated through platinum electrodes at a frequency of 0.5 Hz with rectangular pulses of 0.05 ms duration and of strength sufficient to pro-

duce evoked endplate currents. The evoked endplate currents were filtered by a 5-kHz low pass filter and recorded on magnetic tape. The currents were amplified and digitized by a laboratory interface connected to a computer at a digitization rate of 25 kHz. Ten to 20 evoked endplate currents were collected and averaged after alignment at the middle of their rising phase. Evoked endplate currents decayed as a single exponential function according to the following relationship:

$$I(t) = I(0)\exp^{-t/\tau}$$

where $I(t)$ is the current amplitude at time t after the peak, $I(0)$ is the peak current amplitude and τ is the decay constant.

Experiments were carried out at room temperature. Drug solutions were perfused through the tissue bath for 10 min by peristaltic pump, ensuring a complete exchange of solution.

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G.2.0.3 Sciatic nerve-gastrocnemius muscle preparation in the rabbit

PURPOSE AND RATIONALE

Levis et al. (1953), Long and coworkers (1959, 1967, 1969) described the sciatic nerve-gastrocnemius muscle preparation in the rabbit as an *in vivo* model for testing neuromuscular blocking agents.

PROCEDURE

Dutch rabbits, weighing 1–2 kg, are anesthetized with 200 mg/kg of phenobarbital administered slowly into the marginal ear vein. The sciatic nerve is ligated and cut, and a shielded electrode is placed on the peripheral portion of the nerve. The gastrocnemius muscle is freed as completely as possible from surrounding muscles and a thread is attached to the tendon of the muscle. The twitches of the muscle are elicited by supramaximal stimulation and are recorded through a force transducer. The parameters for interrupted tetanic stimulation are 250 c.p.s. with pulse durations of 1 ms at 15 V applied for 0.2 s every 10 s. The test drugs are administered intravenously into the marginal ear vein.

EVALUATION

The force of contraction after injection of various doses of the test drug is compared to the values obtained prior drug administration.

CRITICAL ASSESSMENT OF THE METHOD

The preparation has the advantage of studying the drug effects under conditions similar to clinical use.

MODIFICATIONS OF THE METHOD

There are two other preparations, the **cat soleus muscle** preparation and the **cat tibialis anticus muscle** preparation that very useful. They can be prepared in a method similar to that described for gastrocnemius muscle except that close arterial injection can be made using the tibialis anticus preparation. Also, soleus muscle consists primarily of slow muscle, whereas tibialis anticus is fast muscle. The experimental procedures for these two preparations are described in detail by Brown (1938), Bowman et al. (1962), Salafsky (1968).

Bowman et al. (1988) investigated structure:action relationships among some desacetoxy analogues of pancuronium and vecuronium in the anesthetized **cat**. Blockade of sciatic nerve-induced contraction of the tibialis and soleus muscles, as well as the effects on vagal-induced bradycardia and on sympathetically induced contractions of the nictitating membrane, were studied.

Khuenl-Brady et al. (1990) used anesthetized **cats** to study the effects of two new nondepolarizing neuromuscular blocking drugs. The indirectly evoked twitch tension of the anterior tibialis muscle elicited by supramaximal square-wave stimuli applied to the peroneal nerve was continuously quantitated by means of a force-displacement transducer and recorded. Onset time (from injection of muscle relaxant to maximum depression of twitch tension), duration of action and recovery index were determined.

Muir et al. (1989) used tibialis anterior and soleus muscle/sciatic nerve preparations in anesthetized **cats** and **pigs** to evaluate neuromuscular blocking agents.

Hoppe (1950) used a nerve-muscle preparation in **dogs** to evaluate curarimimetic drugs. Diaphragmatic respiration was recorded directly by means of a light thread from a suture imbedded in the peritoneal aspect of the right hemi-diaphragm. Stimulation of the peripheral end of the sectioned tibial nerve was accomplished by an induced current once every ten seconds. Muscle contraction was recorded from the Achilles tendon being severed just proximal to the calcaneus.

Hughes and Chapple (1976a,b, 1981; Hughes 1984) used nerve-muscle preparations of cats, dogs and rhesus monkeys.

Ono et al. (1990) recorded the twitch tension of the gastrocnemius-soleus muscle in **rats** after stimulation of the distal stump of the tibial nerve under the influence of a centrally acting muscle relaxant.

Cullen et al. (1980) described two mechanical techniques to measure neuromuscular activity in the intact, anesthetized **dog**. Simultaneous stimulation of the dorsal buccal branch of the facial nerve and ulnar nerve was performed and the evoked mechanical muscle responses measured.

Keesey (1988) discussed the use of single-fiber electromyography (SFEMG) by the AAEE minimonograph 33 as an electrodiagnostic approach to defects of neuromuscular transmission.

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G.2.0.4 Evaluation of neuromuscular blockade in anesthetized mice

PURPOSE AND RATIONALE

Electromyographic investigations of the neuromuscular junction are relatively easy to perform in large animal species such as the dog (Cullen et al. 1980). A simple *in vivo* method for the quantitative evaluation of neuromuscular blockade in anesthetized mice has been described by Lefebvre et al. (1992).

PROCEDURE

Female BALB/c mice, 7–9 weeks old, are anesthetized by intraperitoneal administration of 50 mg/kg etomidate or 250 mg/kg mephenesin. Hair is removed from the sciatic area and hindleg by application of a depilatory cream. A lamp is placed 30 cm above the anesthetized animal to maintain a constant body temperature. Two monopolar needle-stimulating electrodes are subcutaneously inserted into the sciatic notch area and two monopolar recording electrodes subcutaneously over the gastrocnemicus belly and in the vicinity of the tendo calcaneus communis, respectively. A ground electrode is inserted under the skin between recording and stimulating electrodes. Stimulation is carried out using a square signal of 0.2-ms duration. The supramaximal stimulation of the sciatic nerve consists of a train of 10 stimuli, lasting 3.3 s with a frequency of 3 Hz. Stimulation intensity is fixed to a value 50% higher than that required to attain the maximal evoked potential response. Two control trains of stimulation are applied before test-drugs administration and then repeated at 1-min intervals until the end of anesthesia which is assessed by reflex movement after pinching the toes or the tail.

Muscle action potentials are recorded using an electromyograph. The low and high cut-off frequencies are set to 16 Hz and 10 kHz, respectively. The electromyogram signal is amplified and plotted on an oscilloscope screen and printed. Evoked active potentials are analyzed using an 8 bits digitizer and the area under the response wave is evaluated for each stimulus (S).

The neuromuscular blocking agents are administered by intraperitoneal route.

EVALUATION

The effects of the test drugs are quantified by measuring the ratio of the fifth response (S5) to the first one (S1) in a given train (S5/S1 response) according to Keesey (1989). These values are almost not influenced by etomidate anesthesia. Neuroblocking agents of competitive type, such as alcuronium, or depolarizing type, such as suxamethonium, decrease this ratio significantly. These effects can be blocked by neostigmine, but not the effect of the snake venom alpha-bungarotoxin.

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Chapter H
Analgesic, anti-inflammatory, and anti-pyretic activity[1]

[1] Reviewed and amended by R. Schleyerbach, contributions to the first edition by K. U. Weithmann and R. R. Bartlett.

H.1 Central analgesic activity

H.1.0.1 General considerations

Pain is a symptom of many diseases requiring treatment with analgesics. Severe pain due to cancer metastases needs the use of strong analgesics, that means opioid drugs. The addiction liability of opioids led to intensive research for compounds without this side effect. Many approaches have been used to differentiate the various actions of strong analgesics by developing animal models not only for analgesic activity but also for addiction liability. Several types of opioid receptors have been identified in the brain allowing *in vitro* binding tests. However, the *in vitro* tests can only partially substitute for animal experiments involving pain. Pain is a common phenomenon in all animals, at least in vertebral animals, similar to that felt by man. Analgesic effects in animals are comparable with the therapeutic effects in man. Needless to say, that in every instance painful stimuli to animals must be restricted as much as possible. Painful stimuli can consist of direct stimulation of the efferent sensory nerves or stimulation of pain receptors by various means such as heat or pressure. The role of endogenous peptides such as enkephalins and endorphins gives more insight into brain processes and the action of central analgesics.

Pain can also be elicited by inflammation. Progress has been made in elucidating the role of various endogenous substances such as prostaglandins and peptides in the inflammatory process. Most of the so called non-steroidal anti-inflammatory agents have also analgesic activity. Lim and Guzman (1968) differentiated between antipyretic analgesics causing analgesia by blocking impulse generation at pain receptors in the periphery while the narcotic analgesics block synaptic transmission of impulses signaling pain in the central nervous system. An old but excellent survey on methods being used to test compounds for analgesic activity has been provided by Collier (1964). Today, the classification into central and peripheral analgesics is definitively too simplified (Bannwarth et al. 1993) but provides a guide for differentiation by pharmacological methods.

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H.1.1 *In vitro* methods for central analgesic activity

H.1.1.1 Survey

In 1973, high-affinity stereospecific binding of radiolabeled opiate compounds by CNS membrane preparations was reported (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973). The *in vivo* pharmacological potency of opiate agonists and antagonists parallels the *in vitro* displacement of ^{3}H-naloxone, a potent narcotic antagonist. Based on these findings, the ^{3}H-naloxone binding assay was introduced for evaluation of potential analgesics with opiate-like properties. According to different pharmacological profiles of opiates, several receptor types have been identified designated as μ, κ, δ, and σ receptor.

For the **μ receptor, subtypes named μ_1 and μ_2** have been described (Fowler and Fraser 1994; Traynor 1994). Analgesia is thought to involve activation of μ receptors (largely at supraspinal sites) and κ receptors (principally within the spinal cord); δ receptors may also be involved at the spinal and supraspinal level. Other consequences of μ activation include respiratory depression, miosis, reduced gastrointestinal motility, and euphoria. The μ_1 receptors are postulated to mediate the supraspinal analgesic action and the μ_2 receptors to mediate respiratory depression and suppression of gastrointestinal motility. Moreover, different effects on heart rate were described (Paakkari et al. 1992). Two endogenous peptides were described, named **endomorphins**, as agonists with high specific affinity for the μ-receptor (Hackler et al. 1997; Zadina et al. 1997, 1999).

Several studies provide evidence for the existence of **δ-opioid receptor subtypes** (Sofuoglu et al. 1991; Porreca et al. 1992; Horan et al. 1993; Miyamoto et al. 1993; Tiseo and Yaksh 1993). Binding studies with δ opioid receptors have been performed by Mosberg et al. (1983). Endogenous ligands for δ receptors are **enkephalins**.

A rat **κ opioid receptor** has been cloned (Meng et al. 1993). Evidence for different subtypes of the κ-opioid receptor is available (Zukin et al. 1988; Clark et al. 1989; Rothman et al. 1989, 1992, 1993; Wollemann et al. 1993). Endogenous ligands for κ receptors are **dynorphins**.

With the development of highly selective ligands it has become possible to label selectively each of the μ-, δ-, and κ-opioid binding sites.

The **μ-binding sites** are labeled with [^{3}H]-[Tyr-D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (Kosterlitz and Paterson 1981), ^{125}I-FK 33-824 (Moyse et al. 1986), [^{3}H]-Tyr-Pro-MePhe-D-Pro-NH_2 (PL O17; Hawkins et al. 1987), or [^{3}H]-[H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2] (CTOP); Hawkins et al. 1989), the **δ-binding sites** with [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin (Akiyama et al. 1985; Cotton et al. 1985; Mosberg et al. 1987); [^{3}H]-D-Ser^2 (O-*tert*-butyl),Leu^5]enkephalyl-Thr^6 (Delay-Goyet et al. 1988) or [^{3}H]-[D-Pen^2-pClPhe^4,D-Pen^5]enkephalin (Vaughan et al. 1989); [^{3}H]TIPP (Nevin et al. 1993), and the **κ-sites** with [^{3}H]-U-69 593 (Lahti et al. 1985; Maguire et al. 1992), [^{3}H]-PD117 302 (Clark et al. 1988), or [^{3}H]-CI-977 (Boyle et al. 1990) or [^{3}H]norBNI (Marki et al. 1995).

Cloning and molecular biology of opioid receptors has been reviewed (Reisine and Bell 1993).

The opioid receptors were reclassified according to recommendations of IUPHAR (Dhawan et al. 1996, 1998; Alexander and Peters 2000). This nomenclature applies an abbreviation of the generic term for the family (OP for opioid) and a subscript number. **OP_1 stands for δ, OP_2 for κ, and OP_3 for μ receptor.**

OTHER RECEPTORS

There is some evidence that other opioid receptors may exist, such as a β-endorphin-sensitive **ε receptor** (Wüster et al. 1981). The **ζ receptor** (Zagon et al. 1991) and a high affinity binding site referred to as the **λ site** (Grevel et al. 1985) may also be part of the opioid receptor system.

The existence of a **σ receptor** was first postulated by Martin et al. (1976) to account for the psychotomimetic effects of N-allylnormetazocine (SKF 10,047) in the chronic spinal dog. σ Binding sites were proposed to be identical to phencyclidine binding sites based on the finding that phencyclidine generalized to (+)-SKF 10,047 in drug discrimination tests. Further work led to the application of the **term σ to an unique class of non-opiate, non-phencyclidine sites** that may serve as receptors for an as yet unidentified neuromodulator or neurotransmitter (Monnet et al. 1994). At least two subtypes of binding sites, σ_1 and σ_2, are proposed (Bowen et al. 1989; Itzhak and Stein 1991; Karbon et al. 1991; Knight et al. 1991; Connick et al. 1992; Quirion et al. 1992; Leitner et al. 1994). Radioligands for σ receptors (Weber et al. 1986; de Costa et al. 1989) and for subtype σ_1 (Matsuno et al. 1996) and subtype σ_2 (Mach et al. 1999) were described. Pharmacological studies indicate a role of σ receptors not only in analgesia (Mach et al. 1999), but also in motor function (Walker et al. 1993), schizophrenia (Debonnel and

de Montigny 1996; Guitard et al. 1998; Takahashi et al. 1999) and learning and memory (Maurice et al. 1999).

The heterogeneity of opioid receptors has been studied in **isolated tissue preparations** in which neurotransmission is sensitive to inhibition by opioids. The relative potencies of opioid agonists are assessed by their ability to inhibit the electrically evoked contractions of isolated tissue preparations from five different species: the contractions of the mouse **vas deferens** are inhibited by μ-, δ-, and κ-agonists (Maguire et al. 1992), those of **the guinea-pig myenteric plexus-longitudinal muscle** preparation by μ- and κ-agonists (Berzetei-Gurske 1992), those of the **rabbit vas deferens** by κ-agonists, and those of the **hamster vas deferens** by δ-agonists (Sheehan et al. 1986). The contractions of the **rat vas deferens** are inhibited mainly, but not exclusively, by δ-agonists. The actions of β-endorphin in the rat vas deferens are mediated by a further type of opioid receptors, termed **ε-receptor** (Wüster et al. 1981; Corbett et al. 1992; Smith and Leslie 1992).

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H.1.1.2 ^{3}H-Naloxone binding assay

PURPOSE AND RATIONALE

A good correlation between the *in vivo* pharmacological potency of opiate agonists and antagonists with their ability to displace radiolabeled naloxone has been reported. The later discovery that Na^+ (100 mM) enhances the binding of antagonists and reduces the binding of agonists has led to the development of an assay which is used to classify compounds as opiate agonists, mixed agonist-antagonists and antagonists by determining the IC_{50} values for ^{3}H-Naloxone in the presence or absence of Na^+.

PROCEDURE

Reagents

[N-allyl-2,3-^{3}H] Naloxone (38–58 Ci/mmol) is obtained from New England Nuclear.

For IC_{50} determinations ^{3}H-naloxone is made up to a concentration of 100 nM and 50 μl is added to each tube yielding a final concentration 5 nM in the assay.

Levorphanol tartrate is obtained from Hoffmann LaRoche. A stock solution of 1 mM levorphanol is made up in distilled water. This stock is diluted 1 : 200 in distilled water and 20 μl is added to 3 tubes to determine stereospecific binding yielding a final concentration of 0.1 μM in the assay.

Dextrorphan tartrate is obtained from Hoffmann LaRoche. A stock solution of 1 mM dextrophan is made up in distilled water. This stock is diluted 1 : 200 in distilled water and 20 μl is added to the tubes containing the various concentrations of test drug and the tubes for total binding.

Test compounds: For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. At least 7 concentrations are used for each assay. Higher or lower concentrations may be used, depending on the potency of the drug.

Tissue preparation

Male Wistar rats are decapitated and their brains rapidly removed. Whole brains minus cerebella are weighed and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer with a Tekmar tissue homogenizer. The homogenate is centrifuged at 40 000 *g* for 15 min, the supernatant is decanted and the pellet resuspended in fresh buffer and recentrifuged at 40 000 *g*. The final pellet is resuspended in the original volume of fresh 0.05 M Tris buffer. This yields a tissue concentration in the assay of 10 mg/ml.

Assay

310 μl	H_2O
20 μl	5 μM dextrorphan (total binding) or 5 μM levorphanol (non-specific binding)
50 μl	2 M NaCl or H_2O
50 μl	0.5 M Tris buffer, pH 7.7
20 μl	drug or vehicle
50 μl	^{3}H-naloxone
500 μl	tissue suspension.

The tubes are incubated for 30 min at 37 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 3 times with ice-cold 0.05 M Tris buffer, pH 7,7. The filters are then counted in 10 ml of Liquiscint liquid scintillation cocktail. Stereospecific binding is defined as the difference

between binding in the presence of 0.1 µM dextrorphan and 0.1 µM levorphanol. Specific binding is roughly 1% of the total added ligand and 50% of the total bound in the absence of Na^+ and 2% of the total added ligand and 65% of the total bound ligand in the presence of Na^+ (100 mM). The increase in binding is due to an increase in specific binding.

EVALUATION

Data are converted into % stereospecific ^{3}H-naloxone binding displaced by the test drug. IC_{50} values are determined from computer-derived log-probit analysis. The sodium shift is calculated from IC_{50} values with and without NaCl. High sodium shifts are found with agonists, low values with antagonists and medium values with mixed agonists-antagonists.

Data can be analyzed using a computer program as described by McPherson (1985).

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H.1.1.3 ^{3}H-Dihydromorphine binding to µ opiate receptors in rat brain

PURPOSE AND RATIONALE

µ Receptors are considered to mediate the supraspinal activity of opioids. ^{3}H-Dihydromorphine (^{3}H-DHM) exhibits some selectivity for the µ receptor, a high affinity opiate binding site. The test is used to detect compounds that inhibit binding of ^{3}H-DHM in a synaptic membrane preparation obtained from rat brain.

PROCEDURE

Reagents

[1,7,8-^{3}H]Dihydromorphine (^{3}H-DHM) (specific activity 69 Ci/mmol) is obtained from Amersham.

For IC_{50} determinations a 20 nM stock solution is made up. Fifty µl are added to each test tube to yield a final concentration of 0.5 nM in the 2 ml assay.

Levallorphan tartrate is used for the determination of nonspecific binding. A 0.1 mM stock solution is prepared in deionized water. Twenty µl added to each of 3 tubes yields a final concentration of 0.1 µM in the 2 ml assay.

A 1 mM stock solution is made up of the test compounds in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 10^{-6} to 10^{-9} M. At least 7 concentrations are used for each assay.

Tissue preparation

Male Wistar rats are sacrificed by decapitation. Whole brains minus cerebella are removed, weighed and homogenized in 30 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48 000 *g* for 15 min, the supernatant is decanted and the pellet resuspended in the same volume of buffer. This homogenate is then incubated for 30 min at 37 °C to remove the endogenous opiate peptides and centrifuged again as before. The final pellet is resuspended in 50 volumes of 0.05 M Tris buffer, pH 7.7.

Assay

1 850 µl tissue suspension
80 µl distilled water
20 µl vehicle, or levallorphan, or appropriate concentration of drug
50 µl [^{3}H]DHM.

Tubes are incubated for 30 min at 25 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed twice with 5 ml of 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml Liquiscint scintillation cocktail and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 0.1 mM levallorphan. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The K_D value for [^{3}H]DHM binding was found to be 0.38 nM by Scatchard analysis of a receptor saturation experiment. The K_i value may be calculated from the IC_{50} by the Cheng-Prusoff equation:

$$K_i = IC_{50} / 1 + L / K_D$$

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H.1.1.4 ^{3}H-Bremazocine binding to κ opiate receptors in guinea pig cerebellum

PURPOSE AND RATIONALE

κ Receptors are thought to be involved in the analgesic activity of opiates mainly within the spinal cord, whereas μ receptors are predominately located at supraspinal sites. The pharmacological effects of κ agonists differ from the μ agonists in various analgesic tests, effects on diuresis, sensitivity to naloxone and propensity to cause respiratory depression. κ Agonists may induce water diuresis (Salas et al. 1992). The receptor subtype selectivity can be determined by testing the affinity of new compounds for the κ opiate receptor and comparing these results with the data from the μ receptor assay.

Although the benzomorphanes, such as ethylketocyclazocine and bremazocine, are potent κ agonists, they are not selective for this receptor subtype. To demonstrate specific binding of these ligands to κ receptors, the assay must be done in a tissue where the κ subtype predominates, such as the guinea pig cerebellum. Moreover, binding to μ and δ receptors is prevented by inclusion of the peptide DAGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol) to mask the μ receptor and of [D-Pen^{2,5}]-enkephalin (Tyr-D-Pen-Gly-Phe-D-Pen) to mask the δ receptor.

PROCEDURE

Reagents

Bremazocine(–)-[9-^{3}H] (specific activity 21–28 Ci/mmol), is obtained from, New England Nuclear.

For IC_{50} determinations a 24 nM stock solution is made up. Fifty μl are added to each tube to yield a final concentration of 0.6 nM in the 2 ml assay.

U50,488H is made up to a 500 μM stock solution in deionized water. Twenty ml are added to each of the 3 tubes for determination of unspecific binding yielding a final concentration of 5.0 μM in the 2 ml assay.

Opiate peptides of the μ- and δ-type are included in the assay to prevent binding of the radioligand and the test drug to these receptors. DAGO and [D-Pen^{2,5}]-enkephalin are obtained from Penninsula Laboratories. Concentrated stock solutions of 10^{-3} M are made up in deionized water and further diluted to 10^{-5} M. Twenty μl of this solution are added to each tube to result in a final concentration of 100 nM of each in the 2 ml assay.

For the assays a 1 mM stock solution of test compounds is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. At least 7 concentrations are used for each assay.

Tissue preparation

Male guinea pigs are sacrificed and cerebella are removed, weighed and homogenized in 10 volumes of ice-cold 0.05 M Tris-buffer, pH 7.4. The homogenate is centrifuged at 48 000 *g* for 10 min, the supernatant decanted and the pellet resuspended in 20 volumes of buffer. This homogenate is then incubated for 45 min at 37 °C to remove endogenous opiate peptides and centrifuged again as before. This pellet is resuspended in 200 volumes of 0.05 M Tris buffer, pH 7.4.

Assay

1 850 µl tissue suspension
60 µl distilled water
20 µl peptide solution
20 µl vehicle, or U50,488H, or test drug
50 µl [^{3}H]bremazocine

Tubes are incubated for 40 min at 25 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 3 times with 5 ml of 0.05 M Tris buffer. The filters are then placed in scintillation vials with 10 ml Liquiscint scintillation cocktail and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 5.0 µM U50,488H. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The K_D value for [^{3}H]bremazocine binding was found to be 0.14 nM by Scatchard analysis of a receptor saturation experiment. The K_i value may be calculated from IC_{50} by the Cheng-Prusoff equation:

$$K_i = IC_{50} / 1 + L / K_D$$

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H.1.1.5 Inhibition of enkephalinase

PURPOSE AND RATIONALE

Since the discovery of brain peptides with pharmacological properties similar to morphine (Hughes 1975), the metabolic breakdown of enkephalins has been studied (Malfroy et al. 1978; Llorens and Schwartz 1981; Mumford et al. 1981; Malfroy and Schwartz 1982; Roques 1982; Schwartz 1983). Roques BP et al. (1980), Costentin et al. (1986) found that the enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. A highly sensitive fluorometric assay for "enkephalinase", a neutral metalloendopeptidase that releases tyrosine-glycine-glycine from enkephalins has been developed by Florentin et al. (1984). A fluorogenic peptide, dansyl-D-Ala-Gly-Phe(pNO$_2$)-Gly (DAGNPG) was synthesized as a selective substrate for the neutral metalloendopeptidase involved in enkephalin metabolism. This enzyme, designated "enkephalinase" cleaves the Gly-Phe(pNO$_2$) peptide bond of DAGNPG leading to a fluorescence increase related to the disappearance of intramolecular quenching of the dansyl fluorescence by the nitrophenyl residue.

Enkephalinase induces inactivation of atrial natriuretic factor (ANF). The protection of endogenous ANF against inactivation may result in therapeutic applications (Schwartz et al. 1990).

PROCEDURE

Fresh rat kidney is homogenized in 10 vol of cold 0.05 M Tris-HCl buffer, pH 7.4, using a Polytron homogenizer. The homogenate is centrifuged for 5 min at 1 000 g. The pellet is discarded and the supernatant

centrifuged at 60 000 *g* for 60 min. The resulting pellet is resuspended in 50 mM Tris-HCl buffer, pH 7.4, and used as the enzyme source.

Standard assays for "enkephalinase" activity using DAGNPG are carried out at 37 °C in hemolysis tubes. A 0.1-ml amount of 50 mM Tris-HCl buffer, pH 7.4, containing 50 µM DAGNPG is preincubated 15 min at 37 °C. The reaction is initiated by addition of 50 µl of the enzyme preparation together with 0.5 µM Captopril. The tubes are incubated for 30 min in a water bath with constant shaking. The enzymatic reaction is stopped by boiling at 100 °C for 5 min. The samples are then diluted with 1.35 ml of Tris HCl buffer and centrifuged at 500 *g* for 30 min. An aliquot of 1 ml of the supernatant is transferred to thermostated cells of a spectrofluorometer. Readings are performed at 562 nm with an excitation wavelength of 342 nm. A calibration curve is prepared by adding increasing concentrations of DNS-D-Ala-Gly and decreasing concentrations of the substrate in Tris-HCl buffer containing the denaturated enzymatic preparation. For the assay of "enkephalinase" inhibition, the test compound or the standard thiorphan = [(*R*-,*S*-)-3-mercapto-2-benzylpropanoyl]glycine is added in various concentrations.

MODIFICATIONS OF THE METHOD

The inhibitory potencies of test compounds are compared with the standard.

MODIFICATIONS OF THE METHOD

Ksander et al. (1989) incubated synaptic membranes from rat striatum with ^{3}H-Tyr-Leu-enkephalin for 15 min at 30 °C, pH 6.5, in the presence of 10^{-6} M bestatin. The reaction was stopped by the addition of 30% acetic acid and the reaction product ^{3}H-Tyr-Gly-Gly was separated from unreacted ^{3}H-Tyr-Leu-enkephalin on a Porapak Q column followed by a Cu^{2+} chelex column. The ^{3}H-Tyr-Gly-Gly was counted by liquid scintillation.

The antinociceptive effects of intrathecally administered SCH32 615, an enkephalinase inhibitor were studied in the rat by Oshita et al. (1990).

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H.1.1.6 Nociceptin

H.1.1.6.1 General considerations on nociceptin

PURPOSE AND RATIONALE

A heptadekapeptide (nociceptin or orphanin FQ) has been isolated as endogenous agonist of the **opioid receptor-like ORL$_1$ receptor** (Reinscheid et al. 1995; Meunier et al. 1995) which shows high structural homology with opioid peptides, especially dynorphin A (Calò et al. 2000). Nociceptin activates a specific receptor which has been cloned in man and animals and has been shown to be structurally similar to opioid receptors (Mollereau et al. 1994; Calò et al. 2000; Hawkinson et al. 2000). At the cellular level, the nociceptin receptor has been shown to act through the same mechanisms as classical opioid receptors, namely the inhibition of adenylyl cyclase, the activation of potassium

channels and inhibition of calcium channels (Connor et al. 1996a,b). *In vitro* and *in vivo* studies have demonstrated that nociceptin mediates a variety of biological actions (Civelli et al. 1998; Darland et al. 1998). Nociceptin induces analgesia when administered intrathecally (Stanfa et al. 1996; Xu et al. 1996), while it causes hyperalgesia and reversal of opioid induced analgesia when given intracerebroventricularly; nociceptin stimulates food intake (Polidori et al. 2000) and produces anxiolysis. Depending on the dose, nociceptin stimulates or inhibits locomotor activity. Nociceptin inhibits long-term potentiation, memory processes, induces bradycardia, hypotension and diuresis. In addition, nociceptin inhibits neurotransmitter release both at central and peripheral sites. Intracavernosal injection of nociceptin induces a potent and relatively long-lasting erectile response in the cat (Champion et al. 1997, 1998). Intrathecal injection of nociceptin elicits scratching, licking and biting in mice (Sakurada et al. 1999, 2000). Synthetic agonists and antagonists of the nociceptin receptor have been reported (Guerrini et al. 1998; Salvadori et al. 1999; Calò et al. 2000; Hashimoto et al. 2000; Meunier 2000; Ozaki et al. 2000).

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H.1.1.6.2 Receptor binding of nociceptin

PURPOSE AND RATIONALE

The nociceptin receptor has been termed by different groups of investigators as ORL1, LC132, ROR (see Meunier 1997). Based on the structural and transductional similarities between receptors for nociceptin and those for opioids, Hamon (1998) proposed to include the nociceptin receptor in the opioid receptor family with the name OP_4.

Varani et al. (1999) tested synthetic nociceptin analogs for their displacement at the nociceptin- and at classical opioid receptors. The displacement of [^{3}H]$NCNH_2$ ([^{3}H]nociceptin amide, ORL1 site), and of the selective opioid receptor ligands [^{3}H]DAMGO (μ site), [^{3}H]deltorphin II (δ site), and [^{3}H]U69 593 (κ site) was studied.

PROCEDURE

Membrane preparation

Guinea pigs are decapitated and the whole brain (without cerebellum) rapidly removed. The tissue is disrupted in a Polytron homogenizer (setting 5) in 50 mM Tris HCl, pH 7.4, to prepare membranes for the classic opioid receptor studies. The homogenate is centrifuged at 4 000 *g* for 10 min and the pellet is resuspended with a Polytron PTA 10 probe (setting 5) in the same ice-cold buffer. To study the binding to ORL1 receptor, the tissue is homogenized in 50 mM Tris HCl, 2 mM EDTA and 100 μM phenylmethylsulphonylfluoride HCl (PMSF) at pH 7.4. The suspension is centrifuged at 40 000 *g* for 10 min and the pellet is resuspended in the same buffer. After 30 min of incubation at 37 °C the membranes are centrifuged at 40 000 *g* for 10 min and the pellets are stored at –70 °C. The protein concentration is determined with bovine albumin as standard.

Binding assays

Classic opioid receptors, μ, δ and κ, are studied according to Bhargava and Zhao (1996). Saturation binding experiment are carried out using 8–10 different concentrations of [^{3}H]DAMGO ranging from 0.15 nM to 15 nM, [^{3}H]deltorphin II from 0.1 nM to 10 nM, and [^{3}H]U69 593 from 0.15 nM to 15 nM, respectively. Inhibition experiments are carried out in duplicate in a final volume of 250 μl in test tubes containing either 1.5 nM [^{3}H]DAMGO or 1.0 nM [^{3}H]deltorphin II or 1.5 nM [^{3}H]U69 593, 50 mM Tris HCl at pH 7.4, guinea pig brain membranes (150–200 μg of protein/assay) and at least 8–10 different concentrations of the ligands under study. Binding assays to the ORL1 receptor are carried out according to Varani et al. (1998). In saturation studies, membranes are incubated with 8–10 different concentrations of [^{3}H]$NCNH_2$ ([^{3}H]nociceptin amide) ranging from 0.1 mM to 10 mM. Inhibition experiments are carried out in duplicate in a final volume of 250 μl in test tubes containing 1 mM [^{3}H]$NCNH_2$, 50 mM Tris HCl, 2 mM EDTA, 100 μM phenylmethylsulphonylfluoride HCl (PMSF) at pH 7.4, guinea pig membranes, and at least 8–10 different concentration of the compound under examination. The incubation time is 1 h for [^{3}H]DAMGO and [^{3}H]U69 593 and 2 h for [^{3}H]deltorphin II and [^{3}H]$NCNH_2$. Nonspecific binding is defined as the binding measured in the presence of 100 μM bremazocine for classic opioid receptors and 10 μM $NCNH_2$ for ORL1 receptors.

Bound and free radioactivity are separated by filtering the assay mixture through Whatman GF/B glassfibre filters, previously treated with PEI 0.1%; the incubation mixture is diluted with 3 ml of ice-cold incubation buffer, rapidly filtered by vacuum, and the filter washed three times with 3 ml of incubation buffer. The filter-bound radioactivity is measured in a Beckman LS-1 800 Spectrometer.

EVALUATION

The inhibitory binding constant (K_i) values are calculated from the IC_{50} values according to the Cheng and Prusoff equation. The weighted non-linear last-squares curved fitting program LIGAND (Munson and Rodbard 1980) is used for computer analysis of saturation and inhibition experiments.

MODIFICATIONS OF THE METHOD

Seki et al. (1999) analyzed the pharmacological properties of κ-opioid receptor-selective agonist TRK-820 using Chinese hamster ovary cells expressing cloned rat μ-, δ- and κ-opioid receptors and human nociceptin receptor.

Mouledous et al. (2000) reported a site-directed mutagenesis study of the ORL1 receptor transmembrane-binding domain.

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H.1.1.6.3 Bioassays for nociceptin

PURPOSE AND RATIONALE

Nociceptin receptors in the periphery can be characterized by studies in isolated organs (Guerrini et al. 1998; Bigoni et al. 1999): the guinea pig ileum according to Paton (1957) (see J.4.3.1), the mouse vas deferens according to Hughes et al. (1975), the rabbit vas deferens according to Oka et al. (1980) (see A.1.2.3), the guinea pig renal pelvis (Giuliani and Maggi 1996) (see C.4.2.1).

PROCEDURE

Tissues are taken from male Swiss mice (25–30 g), guinea pigs (300–350 g) Sprague Dawley rats (300–350 g) and New Zealand albino rabbits (1.5–1.8 kg). They are suspended in 10 ml organ baths containing Krebs solution oxygenated with 95% O_2 and 5% CO_2. The temperature is set at 33 °C for the mouse vas deferens and at 37 °C for the other tissues. A resting tension of 0.3 g is applied to the mouse deferens, 1 g to the guinea pig ileum, rats vas deferens, and rabbit vas deferens and 0.15 g to the guinea pig renal pelvis. For experiments at the mouse vas deferens a Mg^{2+}-free Krebs solution is used and for rat vas deferens experiments a Krebs solution containing 1.8 mM $CaCl_2$. Guinea pig renal pelvis experiments are performed in the presence of indomethacin (3 μM).

The mouse vas deferens, guinea pig ileum, rat vas deferens, and rabbit vas deferens are continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 ms duration and 0.1 Hz frequency. The electrically evoked contractions are measured isotonically with a strain gauge transducer and recorded on a multichannel chart recorder. After an equilibration period of about 60 min the contractions induced by electrical field stimulation are stable; at this time, cumulative concentration response curves to nociceptin or opioid peptides are performed (0.5 log unit steps).

The guinea pig renal pelvis is stimulated through two platinum ring electrodes with 100 V square wave pulses of 1 ms duration at a frequency of 5 Hz for 10 s. The spontaneous activity and the positive inotropic responses to electrical field stimulation are measured by an isotonic transducer and recorded by a two channel recorder. The experiments are started following a 60 min equilibration period. Four electrical field stimulation are performed with each tissue at 30 min intervals. Agonists are added to the bath 5 min, and antagonists 15 min before the next stimulus. The contractile responses to electrical field stimulation are expressed as % increment the spontaneous activity of the tissue; the biological effects of the application of agonists or antagonists are expressed as % inhibition of electrical filed stimulation-induced contraction.

EVALUATION

Data are expressed as means ±SEM of *n* experiments and statistically analyzed with Student two-tailed *t*-test of one way ANOVA plus Dunnett test. The agonist potencies are given as pE_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. The E_{max} is the maximal effect that an agonist can elicit in a given preparation. Antagonist potencies are expressed in terms of pA_2, which is the negative logarithm to base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration to elicit the original submaximal response.

MODIFICATIONS OF THE METHOD

Rizzi et al. (1999) studied nociceptin and nociceptin analogs in the isolated mouse colon.

Bigoni et al. (1999) used nociceptin, a series of nociceptin fragments, naloxone as well as [$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]nociceptin(1-13)$NH_2$ and [$Nphe^1$]nociceptin (1-13)NH_2 to characterize nociceptin receptors in peripheral organs, such as mouse and rat vas deferens (noradrenergic nerve terminals), in the guinea pig ileum (cholinergic nerves) and renal pelvis (sensory nerves) and *in vivo* by measuring the blood pressure and heart rate in anesthetized rats.

Menzies et al. (1999) described the agonist effects of nociceptin and [$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]nociceptin (1-13)$NH_2$ in the mouse and rat colon and in the mouse vas deferens.

Kolesnikov and Pasternak (1999) found an ED_{50} of 16.3 μg after peripheral administration of nociceptin in the tail flick test in mice.

Bertorelli et al. (1999) found anti-opioid effects of nociceptin and the ORL1 ligand [$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]nociceptin(1-13)$NH_2$ in the Freund's adjuvant-induced arthritic rat model of chronic pain.

Yamamoto and Sakashita (1999) studied the effect of nocistatin, a 17 amino acid peptide which is processed from prepronociceptin and its interaction with nociceptin in the rat formalin test.

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H.1.1.7 Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP)

PURPOSE AND RATIONALE

Several peptides are considered to play a role in the altered transmission of sensory information in neuropathic conditions, such as neuropathic pain arising from trauma or compression injury of peripheral nerves (Zhang et al. 1998).

Vasoactive intestinal polypeptide (VIP), isolated by Nakajima et (1970), is a neuropeptide of 28 amino acids with widespread distribution in both the central and peripheral nervous system (Fahrenkrug 1979; Gafvelin 1990).

Together with the structurally related pituitary adenylate cyclase-activating peptide (PACAP) this peptide is considered to play an important role in the somatosensory processing of pain (Dickinson and Fleetwood-Walker 1999). PACAP-38 (a 38 amino-acid polypeptide) and the C-terminally truncated form PACAP-37 share 68% amino acid homology at their N-terminal domain with VIP. A shorter peptide with 27 amino acids, named as PACAP27 was described by Miyata et al. (1990). These peptides are members of a superfamily of hormones that includes glucagon, glucagon-like peptide, secretin and growth hormone releasing factor.

Three G-protein-coupled receptors are described: the $VPAC_1$ receptor, originally described as the VIP receptor and subsequently designated as VIP_1 receptor; the $VPAC_2$ receptor, previously designated VIP_2; and the PAC_1 receptor, previously known as PACAP type I receptor (Buscail et al. 1990; Guijarro et al. 1991; Felley et al. 1992; Calvo et al. 1994; Van Rampelbergh et al. 1996; Harmar et al. 1998; Robberecht et al. 1999).

Many peripheral activities of VIP/PACAP are described, such as stimulation of pancreatic secretion (Onaga et al. 1997; Ito et al. 1998; Soo Tek Lee et al. 1998); stimulation of duodenal bicarbonate secretion (Takeuchi et al. 1998); relaxation of smooth muscle cells in the intestinal tract, e.g., gall bladder (Pang and Kline 1998), cecal circular smooth muscle (Motomura et al. 1998), internal anal sphincter (Rattan and Chakder 1997); on duodenal motility (Onaga et al. 1998); enhancement of insulin secretion (Yada et al. 1997; Filipsson et al. 1998); bronchodilation (Linden et al. 1998; Shigyo et al. 1998; Okazawa et al. 1998). Centrally administered PACAP showed an anorectic effect (Mizuno et al. 1998).

Agonists (Gourlet et al. 1997a,b) and antagonists (Gozes et al. 1991; Gourlet et al. 1997c) for VIP were described. Further studies are aimed to development of drugs for neuropathic analgesia, ultimately of non-peptide nature, using $VPAC_1$, $VPAC_2$, and PAC_1, receptors as drug targets (Dickinson and Fleetwood-Walker 1999).

PROCEDURE

CHO cell lines expressing the rat VIP_1 receptor (Ciccarelli et al. 1994), the human VIP_2 receptor (Sreedharan et al. 1993), the rat PACAP I receptor (Ciccarelli et al. 1995), and the rat secretin receptor (Ishihara et al. 1991) are used.

Transfected CHO cells are harvested with a rubber policeman and pelleted by low speed centrifugation.

The supernatant is discarded and the cell lysed in mM $NaHCO_3$ solution and immediate freezing in liquid nitrogen. After thawing, the lysate is first centrifuged at 4 °C for 10 min at 400 *g* and the supernatant is further centrifuged at 20 000 *g* for 10 min. The pellet, resuspended in 1 mM $NaHCO_3$ is used immediately as a crude membrane fraction.

Binding is performed using [^{125}I]VIP (specific radioactivity of 0.5 Ci/nmol), [^{125}I]Tyr^{25} secretin (specific radioactivity of 1.0 Ci/nmol) and [^{125}I-Ac-His^{1}] PACAP-27 (specific radioactivity of 0.7 Ci/nmol) as tracers. In all cases, non-specific binding is defined as the residual binding in the presence of 1 μM of the unlabeled peptide corresponding to the tracer. Binding is performed at 37 °C in a 20 mM Tris-maleate, 2 mM $MgCl_2$, 0.1 mg/ml bacitracin, 1% bovine serum albumin (pH 7.4) buffer. Bound radioactivity is separated from free by filtration through glass-fibre GF/C filters presoaked for 24 h in 0.1% polyethyleneimine and rinsed three times with a 20 mM (pH 7.4) sodium phosphate buffer containing 1% bovine serum albumin.

EVALUATION

The IC_{50} values (in mM) for each peptide on each receptor are calculated from complete dose-effect curves performed on three different membrane preparations using the LIGAND program.

MODIFICATIONS OF THE METHOD

Schmidt et al. (1993) studied the binding of PAPAC, VIP and analogues of VIP and PAPAC in rat AR 4-2J pancreatic carcinoma cells and isolated pancreatic acini to the PAPAC-1 receptor, abundantly expressed in AR 4-2J pancreatic carcinoma cells, and to the VIP/PAPAC-2 receptor. Simultaneously, biological effects (lipase secretion and cAMP production) in pancreatic acini were determined. PAPAC was regarded as a potent ligand for both receptor types and as a potent VIP-like secretagogue.

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H.1.1.8 Cannabinoid activity

H.1.1.8.1 General considerations on cannabinoids

In the centuries since hashish and marijuana (*Cannabis sativa*) were used as psychoactive drugs the most significant discoveries in regard to the mechanism of action were made with the isolation of (−)-trans-Δ^9-tetrahydrocannabinol (Δ^9-THC) as the principal active ingredient (Mechoulam et al. 1970), the characterization and localization of the cannabinoid receptor in the brain (Devane et al. 1988), the cloning of its gene (Matsuda et al. 1990), and the identification of an endogenous ligand (Devane et al. 1992). Most cannabinoid effects occur receptor mediated in the CNS (Martin 1986; Herkenham et al. 1990). The recognized CNS responses to cannabinoids include alterations in cognition and memory, euphoria and sedation (Howlett 1995). Cannabinoids have been shown to produce analgesia without the respiratory problems associated with opioid analgesics (Buxbaum 1972; Martin 1985; Dewey 1986; Razdan 1986; Compton et al. 1992; Meng et al. 1998; Strangman et al. 1998) which may be of value for therapeutic applications (Hollister 1986; Izzo et al. 2000a; Pertwee 2000). Simultaneous administration of cannabinoid receptor agonists and μ- or κ-receptor agonists indicate a cannabinoid-opioid interaction in anti-nociception (Manzanares et al. 2000). Baker et al. (1990, 2000) described experimental allergic encephalomyelitis with relapsing-remitting episodes, spasticity and tremor similar to multiple sclerosis in human beings in Biozzi AB/H mice. These symptoms could be antagonized by cannabinoids.

A multiple-evaluation paradigm of *in vivo* mouse assays is employed to test for cannabimimetic effects. This paradigm includes assays for reduction in spontaneous activity, and the production of hypothermia, catalepsy, and antinociception measured by tail-flick assay (Compton et al. 1992; Welch et al. 1998). The behavioral effects of Δ^9-THC and related cannabinoids in mice have been termed the "popcorn" effect. That is, groups of mice are in a sedated state with little or no movement until a stimulus causes one mouse to jump (hyper-reflexia). This animal falls on another mouse which in turn jumps so that this repeated hyper-reflexic jumping looks like corn popping in a machine. Subsequently, all mice will be sedated until another stimulus reinitiates the process (Dewey 1986). Like the opioids, cannabinoids inhibit electrically evoked contractions of the mouse vas deferens and the guinea pig ileum,

but unlike the opioids, these effects are not antagonized by naloxone (Pertwee et al. 1992; Hillard et al. 1999).

In addition to the effects in the CNS, peripheral effects of cannabinoids are known (Lynn and Herkenham 1994) including actions on the **endocrine system** (Patra and Wadsworth 1990, (Block et al. 1991; Wenger et al. 2000), on the **digestive tract** (Rosell and Agurell 1975; Izzo et al. 1990a,b, 2000; Coutts et al. 2000), on **ingestive behavior** (Giuliani et al. 2000), on the **pulmonary and cardiovascular system** (Stengel et al. 1998; White and Hiley 1998; Niederhoffer and Szabo 1999; Liu et al. 2000), and on **immune modulation** (Kaminski et al. 1992; Lynn and Herkenham 1994; Achiron et al. 2000).

An endogenous cannabinoid was isolated from porcine brain by Devane et al. (1992) and found to be an unsaturated fatty acid ethanolamide, arachidonylethanolamide, also called **anandamide**, which activates CB1 receptors (Devane et al. 1992) and produces similar effects as Δ^9-tetrahydrocannabinol including anti-nociception, hypothermia, hypomotility and catalepsy in mice (Smith et al. 1994). The brain enzyme hydrolyzing and synthesizing anandamide has been characterized by Ueda et al. (1995).

Similar effects are produced by other polyunsaturated N-acetylethanolamines, such as N-palmitoylethanolamine, which activates the CB-2-like receptor subtype (Hanu et al. 1993; Facci et al. 1995). Both endogenous cannabinoids (called endocannabinoids) derive from cleavage of a precursor phospholipid, N-acylphosphatidylethanolamine, catalyzed by Ca^{2+}-activated D-type phosphodiesterase activity (Cadas et al. 1996). 2-Arachidonylglycerol was described as a further endogenous ligand for cannabinoid receptors (Ameri and Simmet 2000; Sigiura et al. 2000)

Numerous synthetic analogs and cannabimimetic compounds have been evaluated as agonists and antagonists by *in vitro* and *in vivo* pharmacological methods (Martin et al. 1991; D'Ambra et al. 1992; Melvin et al. 1993; Barth and Rinaldi-Carmona 1999; Hillard et al. 1999).

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H.1.1.8.2 Receptor binding of cannabinoids

PURPOSE AND RATIONALE

After the discovery of cannabinoid receptors in brain (Howlett et al. 1988; Devane et al. 1988; Pertwee 1993), two cannabinoid receptor subtypes were identified: CB1 and CB2. Cannabinoid receptors and were reviewed by Felder and Glass (1998), Pertwee (1999, 2001).

CB1 has an amino acid sequence consistent with a tertiary structure typical of the seven transmembrane-spanning proteins that are coupled to G proteins (Gerard et al. 1990, 1991; Howlett et al. 1990; Matsuda et al. 1990). The CNS responses to cannabinoid compounds are apparently mediated exclusively by CB1, since CB2 transcripts could not be found in brain tissue. CB1 transduces signals in response to CNS active constituents of *Cannabis sativa*, as well as synthetic bicyclic and tricyclic analogs, aminoalkylindole, and eicosanoid cannabimimetic compounds. CB1 is coupled to G_1 to inhibit

adenylate cyclase activity and to a pertussis-sensitive G protein to regulate Ca^{2+} currents. Zimmer et al. (1999) produced a mouse strain with a disrupted CB1 gene. These CB1 knockout mice had a significantly increased mortality rat and displayed reduced locomotor activity, increased ring catalepsy, and hypoalgesia in hot plate and formalin tests.

CB2, the second cannabinoid-binding seven-transmembrane spanning receptor, exhibits 68% identity to CB1 within the helical regions, and 44% identity throughout the total protein. The CB2 clone was derived from a human promyelocytic leukemia cell lin HL60 cDNA library (Munro et al. 1993), also expressed in human leukocytes (Bouaboula et al. 1993). The gene for the rat CB2 receptor was cloned, expressed, and its properties compared with those of mouse and human CB2 receptors (Griffin et al. 2000).

Receptor binding of cannabinoids in correlation to *in vivo* activities was described by Compton et al. (1993).

PROCEDURE

Membrane preparation

Male Sprague Dawley rats weighing 150–200 g are decapitated and the brain rapidly removed. The cortex is dissected free using visual landmarks following reflection of cortical material from the midline and immersed in 30 ml of ice-cold centrifugation solution (320 mM sucrose, 2 mM Tris-EDTA, 5 mM $MgCl_2$). The process is repeated until the cortices of five rats are combined. The cortical material is homogenized with a Potter-Elvehjem glass-Teflon grinding system. The homogenate is centrifuged at 1 600 *g* for 15 min, the supernatant saved and combined with the two subsequent supernatants obtained from washing and 1 600 *g* centrifugation of the P_1 pellet. The combined supernatant fractions are centrifuged at 39 000 *g* for 15 min. The P_2 pellet is resuspended in 50 ml buffer (50 mM Tris-HCl, 2 mM Tris EDTA, 5 mM $MgCl_2$, pH 7.0), incubated for 10 min at 37 °C, then centrifuged at 23 000 *g* for 10 min. The P_2 membrane is resuspended in 50 ml of buffer A, incubated again except at 30 °C for 40 min, then centrifuged at 11 000*g* for 15 min. The final wash-treated P_2 pellet is resuspended in assay buffer B (50 mM Tris-HCl, 1 mM Tris EDTA, 3 mM $MgCl_2$, pH 7.4) to a protein concentration of approximately 2 mg/ml. The membrane preparation is divided into 4 aliquots and quickly frozen in a bath solution of dry ice and 2-methylbutane and then stored at -80 °C.

Binding assay

Binding is initiated by the addition of 150 mg of P_2 membrane to test tubes containing [^{3}H]CP-55,940 (79 Ci/mmol), a cannabinoid analog, (for displacement studies) and a sufficient quantity of buffer C (50 mM Tris-HCl, 1 mM Tris EDTA, 3 mM $MgCl_2$, 5 mg/ml BSA) to bring the total incubation volume to 1 ml. The concentration of [^{3}H]CP-55,940 in displacement studies is 400 pM, whereas that in saturation studies varies from 25 to 2500 pM. Nonspecific binding is determined by the addition of 1 mM unlabeled CP-55,940. The standard CP-55,940 and other cannabinoid analogs are prepared in suspension buffer C from a 1 mg/ml ethanolic stock without evaporation of the alcohol.

After incubation at 30 °C for 1 h, binding is terminated by addition of 2 ml ice-cold buffer D (50 mM Tris-HCl, 1 mg/ml BSA) and vacuum filtration through pretreated filters in a 12-well sampling manifold. Reaction vessels are washed once with 2 ml of ice-cold buffer D, and the filters washed twice with 4 ml of ice-cold buffer D. Filters are placed into 20-ml plastic scintillation vials with 1 ml of distilled water and 10 ml of Budget-Solve (RPI Corp., Mount Prospect, IL). After shaking for 1 h, the radioactivity present is determined by liquid scintillation photometry.

EVALUATION

The B_{max} and K_d values obtained from Scatchard analysis are determined via a suitable computer program. Displacement IC_{50} values are determined by unweighted least squares linear regression of log concentration-percent displacement data and then converted to K_I values.

MODIFICATIONS OF THE METHOD

To further characterize neuronal cannabinoid receptors, Thomas et al. (1998) compared the ability of cannabinoid analogs to compete for receptor sites labeled either with [^{3}H]SR141 716A or[^{3}H]CP-55 940.

Rinaldi-Camora et al. (1998) tested the affinity of an antagonist of the CB2 cannabinoid receptor for rat spleen and cloned human CB2 receptors.

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H.1.1.9 Vanilloid (capsaicin) activity

H.1.1.9.1 General considerations on vanilloids

PURPOSE AND RATIONALE

Several authors reviewed the recent development of capsaicin and vanilloid receptors (Holzer P 1991; Bíró et al. 1997; Sterner and Szallasi 1999; Szallasi and Blumberg 1999; Caterina and Julius 2001; Piomelli 2001). Capsaicin was isolated by Thresh (1846). The chemical structure was determined by Nelson (1919). The analgesic use of capsaicin was reviewed by Lembeck (1987). Capsaicin excites a subset of primary sensory neurons with somata in the dorsal root ganglion or trigeminal ganglion. As a general rule, these vanilloid-sensitive neurons are peptidergic, small diameter (50 μm) neurons, giving rise to thin, unmyelinated C fibers. Among sensory neuropeptides, the tachykinin Substance P shows the best correlation with vanilloid sensitivity. Vanilloid-sensitive neurons transmit noxious information (usually perceived as itching or pain) to the CNS, whereas peripheral terminals rare sites of release of a variety of pro-inflammatory neuropeptides. Among irritant compounds acting on primary sensory neurons, capsaicin and related vanilloids are unique in that the initial stimulation by vanilloids is followed by a long lasting refractory state. Neurotoxicity has been observed when capsaicin as given to newborn rats (Jancsó et al. 1977; Nagy and van der Kooy 1983).

Besides capsaicin, several natural vanilloid agonists were described (Jonassohn and Sterner 1997; Liu et al. 1997; Sterner and Szallasi 1999; Mendes et al. 2000). The irritant principle from *Euphorbia resinifera*, named resiniferatoxin, was isolated by Hergenhahn et al. (1975). In several assays, resiniferatoxin and its derivatives are several thousand-fold more potent than capsaicin (Szolcsanyi et al. 1990; Ács et al. 1995), which is explained by specific receptor binding (Szallasi and Blumberg 1990; Ács et al. 1994). Lee et al. (2001) described simplified resiniferatoxin derivatives as potent vanilloid receptor agonists with potent analgesic activity and reduced pungency.

The high affinity of vanilloid receptors argues for the existence of endogenous vanilloids. Hwang et al. (2000), Piomelli (2001) reported a direct activation of capsaicin receptors by products of lipogenases. Pain-inducing substances, such as bradykinin, may activate phospholipase-linked receptors in sensory neurons, mobilizing arachidonic acid from phospholipids and generating 12-HPETE. This lipid second messenger interacts in turn with a cytosolic domain of the VR1 receptor channel, increasing its opening probability and causing the sensory neuron to become depolarized.

The endogenous ligand of CB_1 cannabinoid receptors, anandamide, is also a full agonist at vanilloid VR1 receptors (Zygmunt et al. 1999; Maccarrone et al. 2000; Smart et al. 2000; DePetrocellis et al. 2001). Premkumar and Ahern (2000) showed that activation of protein kinase C activates VR1 channel activity.

The first capsaicin or vanilloid receptor, termed VR1, was cloned by Caterina et al. (1997). Hayes et al. (2000) reported the cloning and functional expression of a human orthologue of rat vanilloid receptor 1. Pharmacological differences between the human and rat vanilloid receptor 1 were observed (McIntyre et al. 2001). VR1 functions as a molecular integrator of painful chemical and physical stimuli including capsaicin, noxious heat and low pH (Tominaga et al. 1998; Michael and Priestley 1999; Davis et al. 2000; Welch et al. 2000). In mice lacking the capsaicin receptor impaired nociception and pain sensation was observed (Caterina et al. 2000).

Vanilloid receptors are differently distributed in the central and peripheral nervous system (Szallasi 1995; Szallasi et al. 1995; Mezey et al. 2000; Ichikawa and

Sugimoto 2001). Bíró et al. (1998) reported characterization of functional vanilloid receptors expressed by mast cells. Biological and electrophysiological data indicate heterogeneity within the vanilloid receptors. Caterina et al. (1999) described a capsaicin-receptor homologue, named vanilloid-receptor-like protein (VRL-1) with a high threshold for noxious heat. A novel human vanilloid receptor-like protein, named VRL-2 was identified and characterized by Delany et al. (2001).

Several vanilloid antagonists were described, such as capsazepine (Bevan et al. 1992; Walpole et al. 1994) or iodo-resiniferatoxin (Wahl et al. 2001).

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H.1.1.9.2 Vanilloid receptor binding

PURPOSE AND RATIONALE

Ács et al. (1994) described [^{3}H]resiniferatoxin binding by the human vanilloid (capsaicin) receptor. Receptor types and species differences of the vanilloid receptor were described by Szallasi et al. (1994, 1996). The rat vanilloid receptor (rVR1) was cloned and stably expressed in HEK293 cells by Jerman et al. (2000). A detailed pharmacological characterization was conducted using the Ca^{2+}-sensitive dye, Fluo3AM in a fluorimetric imaging plate reader (FLIPR). Ross et al. (2001) studied structure-activity relationship for the endogenous cannabinoid, anadamide, and certain of its analogues at vanilloid receptors in transfected CHO cells.

PROCEDURE

Cell culture

Rat vanilloid receptor (rVR1) transfected CHO cells are maintained in MEM Alpha minus media containing 2 mM L-glutamine supplemented with 10% hyclone fetal bovine serum, 350 µg/ml G418 (Sigma-Aldrich), 100 units/ml penicillin and 100 µg/ml streptomycin. Cells are maintained in 5% CO_2 at 37 °C and passed twice a week using non-enzymatic cell dissociation solution. For the radioligand binding assay, cells are removed from flasks by scraping and then frozen as a pellet at –20 °C for up to one month.

Radioligand binding experiments

Assays are performed in DMEM containing HEPES (25 mM) and BSA (0.25 mg/ml). Total assay volume is 500 µl containing 20 µg of cell membranes. Binding is initiated by addition of [^{3}H]resiniferatoxin ([^{3}H]-RTX). Assays are carried out at 37 °C for 1 h, before termination by addition of ice-cold wash buffer (50 mM Tris-buffer, 1 mg/ml BSA, pH 7.4) and vacuum filtration using a 12-well sampling manifold (Brandell cell harvester) and Whatman GF/B filters that have been soaked in wash buffer at 4 °C for at least 24 h. Each reaction is washed 9 times with a 1.5 ml aliquot of wash buffer. The filters are oven-dried and then placed in 5 ml scintillation fluid. Radioactivity is quantified by liquid scintillation spectrometry. Specific binding is determined in the presence of 1 µM unlabelled RTX. Protein assays are performed using a Bio-Rad De Kit. Unlabelled compounds are added in a volume of 50 µl after serial dilution using assay buffer from a 10 mM stock in ethanol or DMSO. [^{3}H]-RTX is also added in a 50 µl volume following dilution in assay buffer.

EVALUATION

The K_D value and B_{max} for [^{3}H]-RTX and the concentration of competing ligands to produce 50% displacement of the radioligand (IC_{50}) from specific binding sites are calculated using GraphPad Prism (GraphPad Software, San Diego). Dissociation constant (K_i) values are calculated using the Cheng and Prussoff equation.

MODIFICATIONS OF THE METHOD

Wardle et al. (1997) used a 96-well plate assay system to characterize pharmacologically the vanilloid receptor in the dorsal spinal cord of the rat.

Hayes et al. (2000) described the cloning and functional expression of a human orthologue of rat vanilloid receptor-1.

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H.1.1.9.3 Evaluation of vanilloid receptor antagonists

PURPOSE AND RATIONALE

Several vanilloid receptor antagonists were described, such as capsazepine (Bevan et al. 1992; Walpole et al. 1994) or iodo-resiniferatoxin (Wahl et al. 2001). Kirschstein et al. (1999) described the inhibition of rapid heat responses in nociceptive primary sensory neurons of rats by vanilloid receptor antagonists.

PROCEDURE

Adult Sprague Dawley rats of both sexes are deeply anesthetized with diethyl ether and rapidly decapitated. The spine is chilled at 4 °C in F12 Dulbecco's modified Eagle's medium saturated with carbogen gas and additionally containing 30 mM $NaHCO_3$, 100 000 units/l penicillin and 100 mg/l streptomycin. Thoracic and lumbar dorsal root ganglions are quickly dissected and freed from connective tissue. Neurons are dissociated in an incubation chamber enriched with carbogen gas at 37 °C using collagenase CLS II (5–10 mg/ml, 10–12 min) and trypsin (0.2–1 mg/ml, 10–12 min) dissolved in F12 medium. After trituration (4–6 times with a Pasteur pipette) neurons are plated in 35-mm culture dishes, which also serve as recording chambers, and stored at 37 °C in a humidified 5% CO_2 atmosphere before used for electrophysiological recordings.

Only round or oval-shaped neurons without any processes are included in the study. The average of the major and the minor diameter is used to measure the size of oval shaped neurons. Whole cell patch-clamp experiments are performed in carbogen gas saturated F12 medium (pH 7.4) at room temperature using an Axopatch 200A amplifier (Axon Instruments) in voltage-clamp mode at a holding potential of –80 mV controlled by pCLAMP6 software. Data are also registered on a chart recorder. Patch pipettes are fabricated from borosilicate glass using a horizontal micropipette puller and filled with a solution containing (in mM) 160 KCl, 8.13 EGTA, 10 HEPES (pH 7.2, $R_{Tip} = 5.3 \pm 0.2$ MΩ, mean ±SE). Cell diameter, cross sectional area, and membrane capacitance are measured, and excitability is tested by depolarizing voltage steps for each neuron. Cells lacking a fast inward current with a reversal potential close to the equilibrium potential of sodium followed by a prolonged outward current are excluded from further investigation. Experiments in current-clamp mode are performed to measure the resting membrane potential of each neuron and to investigate single action potentials elicited by short (3 ms) depolarizing current pulses in neurons that are hyperpolarized by constant current injection resulting in membrane potentials between –70 and –80 mV. Inflections in the repolarizing phase are qualitatively detected as second negative peak in the first derivative (dV/dt) of each action potential; the duration of repolarization is quantitatively assessed by the 10–90% decay time.

Applications of ~50 µl of heated extracellular solution through a puffing system fixed on a micromanipulator is used to elicit heat-evoked currents. Control measurements with a fast temperature sensor (BAT-12, Physitemp; $\tau = 5$ ms) in place of the neurons are made revealing an effective temperature of ~53 °C, a rise time of ~250 ms, and a decay with a time constant of ~20 s. Effects are compared with those of application of the same amount of medium at room temperature. Heat stimuli with or without vanilloid receptor antagonists and control applications at room temperature are repeated 2–10 times, and the elicited currents are averaged. A neuron is considered as heat sensitive when the heat-evoked inward current is significantly greater than any fluctuations caused by superfusion of solution at room temperature. Heating the buffered solution may change its pH, and acid solution of pH 6.2 are known to activate nociceptive dorsal root ganglion neu-

rons (Bevan and Yeats 1991). The pH of a HEPES-buffered solution decreases while heating (e.g., pH 7.1 at 50 °C). In contrast, higher temperatures increase the pH of a $NaHCO_3/CO_2$ buffer, because the solubility of CO_2 is reduced and thus reverses the HEPES effect. The pH of the F12 medium maximally changes in a range of 7.28–7.52 while heating to 50 °C and cooling down to room temperature. The membrane conductance is measured in voltage-clamp mode by hyperpolarizing pulses (5 mV, 10 ms, 50 s^{-1}), and conductance changes are determined at the maximum amplitude of heat evoked currents.

Reversal potentials of heat- and capsaicin-induced currents are measured as described by Liu et al. (1997) using fast depolarizing ramps (–80 to +30 mV in 22 ms every 550 ms). Patch pipettes are filled with a potassium-free solution containing (in mM) 140 CsCl, 10 HEPES, 10 EGTA, and 4 $MgCl_2$ (adjusted to pH 7.2). Tetrodotoxin (100 µM) and nifedipine (1 µM) are added to the extracellular solution to block voltage gated Na^+ and Ca^{2+} channels. Capsaicin is dissolved in ethanol, diluted to its final concentration with F12 medium, and applied through the puffing system. Capsazepine (dissolved in DMSO) and ruthenium red are prepared as concentrated stock solutions, diluted to final concentration in F12 medium, and applied either at room temperature or at ~53 °C. Reversibility of antagonist action is tested by reapplication of heated extracellular solution without any agents.

EVALUATION

Off-line measurements and statistical analysis is done using pCLAMP6 (Axon Instruments) and EXCEL 5.0 (Microsoft). Data are presented as means ±SE. Treatment effects are statistically analyzed by Student's *t*-test for paired data and χ^2 test for analysis of incidences.

MODIFICATIONS OF THE METHOD

Nagy et al. (1983) described dose-dependent effects of capsaicin on primary sensory neurons in the neonatal rat.

Lopshire and Nicol (1998) performed whole-cell and single-channel studies in rat sensory neurons and found a prostaglandin E_2 induced enhancement of the capsaicin elicited current.

Jung et al. (1999) performed patch-clamp experiments in dorsal root ganglion neurons of neonatal rats and concluded that capsaicin binds to the intracellular domain of the capsaicin-activated ion channel.

Nagy and Humphrey (1999) compared the membrane responses of rat sensory neurons to noxious heat and capsaicin, using electrophysiological and ion flux measurements.

Baumann and Martenson (2000) found that extracellular protons both increase the activity and reduce the conductance of capsaicin-gated channels.

Liu et al. (2001) investigated mechanisms underlying capsaicin-mediated inhibition of action potentials and modulation of voltage-gated sodium channels in cultured trigeminal ganglion neurons.

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H.1.2 *In vivo* methods for testing central analgesic activity

H.1.2.1 General considerations

Although the *in vivo* methods have been used more extensively in the past, they are still necessary in present research analgesic tests in animals before a compound can be given to man. Mostly, rodents, such as

mice or rats, are used for analgesic tests, but in some instances experiments in higher animals such as monkeys are necessary.

Several methods are available for testing central analgesic activity, such as

- HAFFNER's tail clip method in mice,
- tail flick or other radiant heat methods,
- tail immersion tests,
- hot plate methods in mice or rats,
- electrical stimulation (grid shock, stimulation of tooth pulp or tail),
- monkey shock titration,
- formalin test in rats.

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H.1.2.2 HAFFNER's tail clip method

PURPOSE AND RATIONALE

The method was described as early as 1929 by Haffner who observed the raised tail (Straub phenomenon) in mice treated with morphine or similar opioid drugs and found the tail after drug treatment to be less sensitive to noxious stimuli. He already described the high sensitivity of this method to morphine. Since then, the method has been used and modified by many authors.

PROCEDURE

An artery clip is applied to the root of the tail of mice and the reaction time is noted. Male mice (Charles River strain or other strains) with a weight between 18 and 25 g are used. The control group consists of 10 mice. The test compounds are administered subcutaneously to fed mice or orally to fasted animals. The test groups and the control group consist of 7–10 mice. The drug is administered 15, 30 or 60 min prior testing. An artery clip is applied to the root of the tail (approximately 1 cm from the body) to induce pain. The animal quickly responds to this noxious stimuli by biting the clip or the tail near the location of the clip. The time between stimulation onset and response is measured by a stopwatch in 1/10 seconds increments.

EVALUATION

A cut-off time is determined by taking the average reaction time plus 3 times the standard deviation of the combined latencies of the control mice at all time periods. Any reaction time of the test animals which is greater than the cut-off time is called a positive response indicative of analgesic activity. The length of time until response indicates the period of greatest activity after dosing. An ED_{50} value is calculated at the peak time of drug activity. ED_{50} values found by this method were 1.5 mg/kg s.c. for morphine and 7,5 mg/kg for codeine s.c.

CRITICAL ASSESSMENT OF THE TEST

The test does not need any sophisticated equipment but a skilled, preferably "blind", observer. Peripheral analgesics of the salicylate type are not detected by this test.

MODIFICATIONS OF THE METHOD

Bartoszyk and Wild (1989) described a modification of the original Haffner clip test using pressure on the tail of rats instead of mice. Additionally hyperalgesia was induced by injection of carrageenan suspension into the tail. In this case not only an effect of a nonsteroidal anti-inflammatory agent but also a potentiation by B-vitamins could be shown.

Takagi et al. (1966) published a modification of HAFFNER's method for testing analgesics.

Ossipov et al. (1988) used the Haffner test to compare the antinociceptive effects of intrathecally administered opiates, α_2-adrenergic agonists, and local anesthetics.

Yanagisawa et al. (1984) described a tail pinch method *in vitro* for testing antinociceptive drugs consisting of an isolated spinal cord, spinal nerve roots and the functionally connected tail of a new-born rat. Changes of electric potential in the ventral root are induced by noxious pressure on the tail. In addition, responses after electric stimulation of the dorsal root were recorded. The authors recommend the method for studying actions of analgesic drugs.

Pinch of the toes of guinea pigs was recommended as a test for opioid analgesics by Collier (1965).

Tail-pinch feeding in rats after intracerebroventricular injection of various opioid antagonists has been used to differentiate opioid receptor subtypes (Koch and Bodnar 1993).

Person et al. (1985) used three different techniques of mechanical tail stimulation (reaction threshold determined with an Analgesy-meter at two different cut-off values and HAFFNER's tail clip) to study morphine-caffeine analgesic interaction in rats.

Arndt et al. (1984) studied pain responses (increase of heart rate and arterial pressure, respiratory effects) to tail clamping in trained unanesthetized spontaneously breathing dogs after administration of fentanyl.

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H.1.2.3 Radiant heat method

PURPOSE AND RATIONALE

Originally, the method was developed by Schumacher et al. (1940), Wolff et al. (1940) for quantitative measurements of pain threshold in man against thermal radiation and for evaluation of analgesic activity of opiates. Later on, the procedure has been used by many authors to evaluate analgesic activity in animal experiments by measuring drug-induced changes in the sensitivity of mice or rats to heat stress applied to their tails. The test is very useful for discriminating between centrally acting morphine-like analgesics and non-opiate analgesics.

Mice are placed into cages leaving the tail exposed. A light beam is focused to the proximal third of the tail. Within a few seconds the animal flicks the tail aside or tries to escape. The time until this reaction occurs is measured.

PROCEDURE

The method was described by Ther, Lindner and Vogel (1963) as a modification of earlier publications (D'Armour and Smith 1941). Groups of 10 mice (NMRI-strain) of both sexes with a weight between 18 and 22 g are used for each dose. Before administration of the test compound or the standard the normal reaction time is determined. The animal is put into a small cage with an opening for the tail at the rear wall. The tail is held gently by the investigator. By opening of a shutter, a light beam exerting radiant heat is directed to the proximal third of the tail. For about 6 s the reaction of the animal is observed by the investigator. The mouse tries to pull the tail away and turns the head. With a switch the shutter is closed as soon as the investigator notices this reaction. Mice with a reaction time of more than 6 s are not used in the test. The escape reaction which is the endpoint of this test can be regarded as a complex phenomenon mediated by the brain. In contrast, the simple tail flick as an endpoint of this test may be mediated as a spinal reflex. Therefore the observation of the escape reaction can be regarded as a true assessment of the influence of the drug on the brain.

The test compounds and the standard are administered either orally or subcutaneously. The animals are submitted to the same testing procedure after 30, 60 and eventually 120 min. For each individual animal the reaction time is noted. Other time intervals can be used according to the question to be investigated.

EVALUATION

There are two possibilities for evaluation:

- The average values of reaction time after each time interval are calculated and compared with the pretest value by analysis of significance.
- At each time interval only those animals which show a reaction time twice as high or higher as the pretest value are regarded as positive. Percentages of positive animals are counted for each time interval and each dose and ED_{50} values are calculated according to LITCHFIELD and WILCOXON.

As standards codeine, pethidine and morphine can be used. The ED_{50} values of these drugs are:

- Codeine 12 mg/kg s.c.
- Pethidine 12 mg/kg s.c.
- Morphine 2 mg/kg s.c.

CRITICAL ASSESSMENT OF THE TEST

The radiant heat test on the tail of mice is very effective to estimate the efficacy and potency of central acting analgesic drugs. With pyrazolones ED_{50} values still can be calculated but these are achieved only with relatively high doses. Compounds like acetylsalicylic acid

and phenyl-acetic acids show only slight effects making it impossible to calculate ED_{50} values.

MODIFICATIONS OF THE METHOD

Originally, the method has been described for testing analgesic properties in the rat (D'Armour and Smith 1941, Winter et al. 1954, Harris and Pierson 1964). Goldstein and Malseed (1979) adapted the procedure for utilization in **cats**. The effect of morphine could be antagonized by naloxone in this test. No response to sodium salicylate or pentobarbital was observed.

Lutz et al. (1994) used a modification of the rat tail withdrawal test to investigate the structure-activity profile of a series of opioid analgesics. One day before testing, polyethylene tubings were implanted in the femoral vein and externalized behind the neck for intravenous application of test substances.

Various instruments have been described for measuring tail flick latencies by several authors, e.g., Davies et al. 1946; Owen et al. (1981), Isabel et al. (1981), Walker and Dixon (1983), Yoburn et al. (1984), Harris et al. (1988).

Tail flick analgesy meters are commercially available (e.g., IITC Life Science, Woodland Hills, CA, USA).

Green and Young (1951) compared the heat and pressure analgesiometric methods in rats. Mohrland et al. (1983) described an ultrasound-induced tail-flick procedure.

Hargreaves et al. (1988), Costello and Hargreaves (1989), Hylden et al. (1991) exposed the plantar surface of hindpaws of unrestrained rats to a beam of radiant heat applied through the glass floor of a testing chamber. Paw withdrawal latency was automatically recorded by a photocell.

This method was also used by Schuligoi et al. (1994).

Taylor et al. (1997) used this method to investigate the brief (phase 1) and persistent (phase 2) nociceptive responses of rats after injection of dilute formalin into the hindpaw.

Carmon and Frostig (1981) used brief laser induced heat applied to the rat ear for pharmacological testing of analgesics.

Perkins et al. (1993), Perkins and Kelly (1993) used ultra-violet-induced hyperalgesia in rat paw. Female Sprague-Dawley rats weighing about 100 g were exposed on the plantar surface of one hind paw to UV light (intensity maximum 365 nm, 69 mW/cm^2) for 90 s and this was repeated 18 h later. On the following days, each group of rats was placed in a transparent Perspex box and the withdrawal threshold to a focused beam of radiant heat applied to the underside of each hind paw was measured.

McCallister et al. (1986) directed radiant heat to the **ears of rabbits** and measured ear-withdrawal time.

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H.1.2.4 Hot plate method

PURPOSE AND RATIONALE

The paws of mice and rats are very sensitive to heat at temperatures which are not damaging the skin. The responses are jumping, withdrawal of the paws and licking of the paws. The time until these responses occur is prolonged after administration of centrally acting analgesics, whereas peripheral analgesics of the acetylsalicylic acid or phenyl-acetic acid type do not generally affect these responses.

PROCEDURE

The method originally described by Woolfe and Mac Donald (1944) has been modified by several investigators. The following modification has been proven to be suitable:

Groups of 10 mice of either sex with an initial weight of 18 to 22 g are used for each dose. The hot plate, which is commercially available, consists of a electrically heated surface. The temperature is controlled for 55° to 56 °C. This can be a copper plate or a heated glass surface. The animals are placed on the hot plate and the time until either licking or jumping occurs is recorded by a stop-watch. The latency is recorded before and after 20, 60 and 90 min following oral or subcutaneous administration of the standard or the test compound.

EVALUATION

The prolongation of the latency times comparing the values before and after administration of the test compounds or the values of the control with the experimental groups can be used for statistical comparison using the *t*-test. Alternatively, the values which exceed the value before administration for 50% or 100% can be regarded as positive and ED_{50} values can be calculated.

Doses of 7.5 mg/kg s.c. morphine hydrochloride, 30 mg/kg s.c. codeine hydrochloride, 30 mg/kg s.c. pethidine hydrochloride and 400 mg/kg s.c. phenazone were found to be effective, whereas aspirin showed no effect even at high doses.

CRITICAL ASSESSMENT OF THE TEST

The hot plate test has been used by many investigators and has been found to be suitable for evaluation of centrally but not of peripherally acting analgesics. Mice as well as rats have been used. The method has the drawback that sedatives and muscle relaxants (Woolfe and MacDonald 1944) or psychotomimetics (Knoll 1967) cause false positives, while mixed opiate agonists-antagonists provide unreliable results. The validity of the test has been shown even in the presence of substantial impairment of motor performance (Plummer et al. 1991). Mixed opiate agonists-antagonists can be evaluated if the temperature of the hot plate is lowered to 49.5 °C (O'Callaghan and Holtzman 1975; Zimer et al. 1986).

MODIFICATIONS OF THE METHOD

O'Neill et al. (1983) described an automated, high-capacity method for measuring jump latencies on a hot plate. A hot-plate test with increasing temperature was recommended by Tjølsen et al. (1991).

Hot plate analgesy meters are commercially available (e.g., IITC Life Science, Woodland Hills, CA, USA).

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H.1.2.5 Tail immersion test

PURPOSE AND RATIONALE

The method has been developed to be selective for morphine-like compounds. The procedure is based on the observation that morphine-like drugs are selectively capable of prolonging the reaction time of the typical tail-withdrawal reflex in rats induced by immersing the end of the tail in warm water of 55 °C.

PROCEDURE

Young female Wistar rats (170–210 g body weight) are used. They are placed into individual restraining cages leaving the tail hanging out freely. The animals are allowed to adapt to the cages for 30 min before testing. The lower 5 cm portion of the tail is marked. This part of the tail is immersed in a cup of freshly filled water of exactly 55 °C. Within a few seconds the rat reacts by withdrawing the tail. The reaction time is recorded in 0.5 s units by a stopwatch. After each determination the tail is carefully dried. The reaction time is determined before and periodically after either oral or subcutaneous administration of the test substance, e.g., after 0.5, 1, 2, 3, 4 and 6 h. The cut off time of the immersion is 15 s. The withdrawal time of untreated animals is between 1 and 5.5 s. A withdrawal time of more than 6 s therefore is regarded as a positive response.

EVALUATION

ED_{50} values can be calculated for each compound and time response curves (onset, peak and duration of the effect) be measured. All the morphine-like analgesics have been shown to be active at doses which do not produce gross behavioral changes. For example, an ED_{50} of 3.5 mg/kg s.c. for morphine and an ED_{50} of 1.7 mg/kg s.c. methadone was found. Acetylsalicylic acid at a dose of 640 mg/kg p.o., phenylbutazone at a dose of 160 mg/kg s.c. as well as nalorphine at a dose of 40 mg/kg s.c. were inactive.

CRITICAL ASSESSMENT OF THE METHOD

The test is useful to differentiate central opioid like analgesics from peripheral analgesics.

MODIFICATIONS OF THE TEST

Ben-Bassat et al. (1959) described the receptacle method in mice. Each mouse was inserted in a conoid paper receptacle with its tail protruding, the cone being closed by a stapler. The protruding tail was entirely immersed in a water bath (58 °C) and the time until withdrawal of the tail was measured by a stop watch.

Pizziketti et al. (1985) modified the tail immersion test in rats in this way that they used a 1 : 1 mixture of ethylene-glycol and water **cooled** to a temperature of minus 10 °C as noxious stimulus. Linear dose-response curves were found with levo-methadone and morphine. Low ceiling effects or curvilinear dose-response curves were obtained with narcotic agonist- antagonist analgesics such as pentazocine. Diazepam and aspirin were inactive.

Tiseo et al. (1988) could show that the endogenous kappa agonist dynorphin A was inactive in the rat tail immersion test at 55 °C, but gave dose-response curves in the cold water version of the test.

Abbott and Melzack (1982) examined the effects of brainstem lesions on morphine analgesia using the formalin test which produced moderate pain that lasted about 2 h, and the tail-flick hot water-immersion test which measured brief threshold-level pain.

Abbott and Franklin (1986) used two forms of the rat tail flick test: In the restrained form of the test rats were placed in wire restraining tubes from 10 min before drug injection till the end of the test. In the unrestrained form of the test rats were left free in their home

cages and handheld during each test for approximately 30 s. Responses to the thermal pain stimulus were assessed by the latency with which the rat removed its tail from 55 °C water. Two types of morphine analgesia have been postulated in animals: One type, exemplified in rats that are restrained during tail flick testing, is sensitive to an interaction between morphine and brain 5-HT, the level of which is elevated by restrained stress (Kelly and Franklin 1984).

Luttinger (1985) determined the antinociceptive activity of drugs using different water temperatures in a tail-immersion test in mice. The results roughly paralleled the differences in the severity of pain for which various analgesics are effective.

Dykstra et al. (1986, 1987) described a tail withdrawal procedure for assessing analgesic activity in **Rhesus monkeys** by immersion of the tail into water of 55 °C. This procedure was used by Rothman et al. (1989) to determine the pharmacological activities of optically pure enantiomers of the κ opioid agonist, U50,488, and its cis diastereomer.

Using this method, Ko et al. (1999) found that activation of peripheral κ opioid receptors inhibits capsaicin-induced nociception in Rhesus monkeys.

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H.1.2.6 Electrical stimulation of the tail

PURPOSE AND RATIONALE

Since the tail of mice is known to be sensitive to any stimulus, a method of electrical stimulation has been described as early as 1950 by Burn et al. The stimulus can be varied either by the duration of the electric shock or by an increase in the electric current.

PROCEDURE

As described by Kakunaga et al. (1966), male mice with a weight of 20 g are placed into special cages. A pair of alligator clips is attached to the tail whereby the positive electrode is placed at the proximal end of the tail. Rectangular wave pulses from a constant voltage stimulator at an intensity of 40–50 V are applied. The frequency of the stimulation is 1 shock/s, and the pulse duration 2.5 ms. The normal response time range of the stimuli is 3–4 s. Following administration of the drug, the response time is registered at 15 min intervals until the reaction time returns to control levels.

EVALUATION

The data for each animal are plotted with reaction times on the ordinate and time intervals following administration on the abscissa. The area under the time response curve is calculated. In control animals the reaction time remains fairly constant and the area under the curve is

approximately zero. Effects of morphine at 5 mg/kg s.c. and meperidine 30 mg/kg s.c. could easily be demonstrated.

CRITICAL ASSESSMENT OF THE METHOD

The effect of central analgesics can be clearly demonstrated, however also the activity of peripheral analgesics given at higher doses can be detected.

MODIFICATIONS OF THE METHOD

Vidal et al. (1982) measured the thresholds of 3 nociceptive reactions (tail withdrawal, vocalization, vocalization afterdischarge) following electrical stimulation of the tail.

A variation of the test has been introduced by Yanaura et al. (1976), using ultrasonic stimulation instead of electric stimulation. The method is considered to be fast, simple, and precise. The stimulus can be applied repeatedly without causing injury to the tissue.

A vocalization test in rats with electrical stimulation of the tail has been described by Hoffmeister (1968).

Ludbrook et al. (1995) described a method for frequent measurement of sedation and analgesia in sheep using the response to a ramped electrical stimulus. Sheep were placed in a canvas sling in their metabolic crates to allow their limbs to partially bear weight in order to minimize spontaneous limb movements. Two needles were placed subcutaneously 0.5 cm apart in the anterior aspect of the lower third of the sheep's hind and connected to the nerve stimulator. The current ramp rate was set at one mA per sec. As soon as limb withdrawal was observed, the stimulus was switched off and the highest current and ramp duration were recorded.

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H.1.2.7 Grid shock test

PURPOSE AND RATIONALE

The electric grid shock test in mice has been described by Blake et al. (1963) as a modification of an earlier approach (Evans 1962) to measure the analgesic properties by the "Flinch-jump" procedure in rats.

PROCEDURE

Male mice with a weight between 18 and 20 g are individually placed into clear plastic chambers. The floor of the box is wired with tightly strung stainless steel wire, spaced about 1 mm apart. The stimulus is given in the form of square wave pulses, 30 cycles per second with a duration of 2 ms per pulse. The output of the stimulator has to be connected to alternate wires of the grid. A fixed resistance is placed in series with the grid and in parallel to an oscilloscope to allow calibration in milliamperes. With increasing shock intensities the mice flinch, exhibit a startling reaction, increase locomotion or attempt to jump. The behavior is accurately reflected on the oscilloscope by marked fluctuations of the displayed pulse and defined as pain threshold response. Pain thresholds are determined in each individual mouse twice before administration of the test drug and 15, 30, 60, 90 and 120 min after dosing. Groups of 10 animals are used for control and for the test drugs.

EVALUATION

The current as measured in milliamperes is recorded for each animal before and after administration of the drug. The average values for each group at each time interval are calculated and statistically compared with the control values. Placebo treated controls show a slight increase of threshold over time. Morphine sulfate in a dose of 10 mg/kg p.o. but also acetylsalicylic acid in a dose of 200 mg/kg p.o. definitely increase the threshold.

CRITICAL EVALUATION OF THE METHOD

The modification of the method as described by Blake et al. (1963) showed an effect not only of morphine but also of acetylsalicylic acid which is not easily picked up by other tests based on stimulation by physical means.

MODIFICATIONS OF THE TEST

Weiss and Laties (1961) in a "fractional escape" procedure trained animals to press a lever to reduce the intensity of shock delivered continuously through the floor grids of the experimental chamber. Each time, the rat depresses the lever, it reduces the intensity of the shock. An external timer is programmed to increase the intensity of the shock every few seconds. If the animal fails to press the lever, the shock continues to increase in intensity until lever-pressing behavior drives it down. Thus, the level of shock fluctuates depending on the rat's lever pressing. The action of an analgesic in altering the level of shock which the rat will "tolerate" can than be measured by comparing the average level at which the rat maintains the shock under control conditions with the average level at which the rat maintains the shock during treatment.

Painful stimulation of the paws of mice placed into cages equipped with metal bands for electrical stimulation was described by Charlier et al. (1961) as "pododolorimetry".

A modification of the jump-flinch technique for measuring pain sensitivity in rats based on four cate-gories of responses was described by Bonnet and Peterson (1975).

Eschalier et al. (1988) described an automated method to analyze vocalization of unrestrained rats submitted to noxious stimuli.

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H.1.2.8 Tooth pulp stimulation

PURPOSE AND RATIONALE

The method has been first described by Kohl and Reffert (1938) and by Ruckstuhl and Gordanoff (1939) for testing central analgesic activity in rabbits and has since applied by several authors to various animal species. Stimulation of the tooth pulp induces characteristic reactions, such as licking, biting, chewing and head flick which can be observed easily.

PROCEDURE

Rabbits of either sex with an weight between 2 and 3 kg are anaesthetized with 15 mg/kg thiopental or 0.2 mg/kg fentanyl-citrate intravenously. Pulp chambers are exposed close to the gingival line in the lateral margins of the two front upper incisors with a high-speed dental drill. On the day of the experiment, clamping electrodes are placed into the drilled holes. After an accommodation period of 30 min stimulation is started to determine the threshold value. The stimulus is applied by rectangular current with a frequency of 50 Hz and a duration of the stimulus of 1 s. The electrical current is started with 0.2 mA and increased until the phenomenon of licking occurs. In some cases, the current has to be increased and than to be decreased again in order to find the appropriate threshold. For assessing the basic value, the threshold is determined 3 times in each animal. Each animal serves as its own control. For testing analgesic activity of a new drug and determination of an ED_{50} 8 to 10 animals are used for each dose of the analgesic. The test substance is either injected intravenously or given orally by gavage. The threshold as the indicator of the antinoci-ceptive effect is determined again after 15, 30, 60 and 120 min. The animals serve as their own controls. Threshold current is determined again 5, 15, 30, 45 and 60 min after intravenous application and 15, 30, 60 and 120 min after oral application.

EVALUATION

For screening procedures the increase of threshold, expressed in mV, is the indicator of intensity and duration of the analgesic effect. For determination of the ED_{50}, 8 to 10 rabbits are used for each dose, using 3 doses, which provided effects between 10 and 90%. An antinociceptive effect is defined as an increase of

the threshold versus the initial control by a factor of 2 or more.

CRITICAL ASSESSMENT OF THE METHOD

Central analgesics, especially opioid agonists, have been found to be very active in this test. Compared with other tests for central analgesic activity, like the hot plate test in mice, the tests result in lower ED_{50}-values indicating a high sensitivity of the method. In addition, non-opiate analgesics like ketamine and peripheral analgesics like pyrazolone derivatives gave a positive response.

MODIFICATIONS OF THE METHOD

The method has been performed primarily in rabbits (Hertle et al. 1957; Hoffmeister 1962, 1968; Piercey and Schröder 1980), but also **dogs** (Koll and Fleischmann 1941; Skinkle and Tyers 1979) and **cats** (Mitchell 1964) have been used.

Among several methods in different species, Fleisch and Dolivo (1953) found the electrical stimulation of the tooth pulp in the rabbit as the only satisfactory method to test the efficacy of different analgesic drugs.

The effects of tooth pulp stimulation in the thalamus and hypothalamus of the **rat** have been investigated by Shigena et al. (1973).

The method has been adapted for freely moving rats (Steinfels and Cook 1986). Medium effective doses could be determined for μ and κ agonists. Non-steroidal anti-inflammatory drugs were also effective in this test procedure, but the slopes of the dose-response curves for these compounds were lower than for the opioid analgesics.

Microinfusion of bradykinin solution onto the tooth pulp of unrestrained rats was described by Foong et al. (1982) as a reliable method for evaluating analgesic potencies of drugs on trigeminal pain.

Kidder and Wynn (1983) described an automatic electronic apparatus for generating and recording a ramp stimulus for analgesia testing.

Thut et al. (1995) used the rabbit tooth-pulp assay to quantify efficacy and duration of antinociception by local anesthetics infiltrated into maxillary tissues.

Shyu et al. (1984) studied the role of central serotoninergic neurons in the development of dental pain in the **monkey**.

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H.1.2.9 Monkey shock titration test

PURPOSE AND RATIONALE

Generally, analgesic tests in rats and mice result in correlation with the analgesic activity of a drug in man. To clarify the mode of action in more detail and to find a suitable dose for therapy in man, experiments in monkeys may be necessary.

PROCEDURE

This test has been recommended by Weiss and Laties (1958) and later developed further by several authors. The monkeys are seated in restraining chairs. Electrical current is delivered by a Coulbourn Instrument Programmable Shocker through electrodes coupled to two test tube clamps which are attached to a shaved portion of the tail. The current ranges from 0 to 4 mA through 29 progressive steps. The monkey presses a bar to interrupt the shock. A stable baseline shock level is established for each monkey on the day prior to drug administration. After drug administration shock titration activity is rated according to the change in maximum level of median shock intensity attained for drug as compared to control levels. Doses of 3.0 mg/kg i.m. morphine, 1.7 mg/kg i.m. methadone and 10 mg/kg i.m. pentazocine were found to be effective.

CRITICAL ASSESSMENT

The monkey shock titration test may be used for final evaluation of a new compound before administration to man. For screening activities the procedure can not be recommended since the test is too time consuming and the apparatus too complicated. Furthermore, higher animals such as monkeys should only be used if absolutely necessary.

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H.1.2.10 Formalin test in rats

PURPOSE AND RATIONALE

The formalin test in rats has been proposed as a chronic pain model which is sensitive to centrally active analgesic agents by Dubuisson and Dennis (1977).

PROCEDURE

Male Wistar rats weighing 180–300 g are administered 0.05 ml of 10% formalin into the dorsal portion of the front paw. The test drug is administered simultaneously either sc. or orally. Each individual rat is placed into a clear plastic cage for observation. Readings are taken at 30 and 60 min and scored according to a pain scale. Pain responses are indicated by elevation or favoring of the paw or excessive licking and biting of the paw. Analgesic response or protection is indicated if both paws are resting on the floor with no obvious favoring of the injected paw.

EVALUATION

Using various doses, ED_{50} values for protection can be calculated. Doses of 1.7 mg/kg morphine s.c. and 15 mg/kg s.c. pethidine were found to be effective.

CRITICAL ASSESSMENT

The formalin test identifies mainly centrally active drugs, whereas peripherally acting analgesics are almost ineffective. Therefore, the formalin test may allow a dissociation between inflammatory and non-inflammatory pain, a rough classification of analgesics according to their site and their mechanism of action (Chau 1989). Cowan (1990) underlined the aspect that the formalin-test is a model of chronic pain whereas most other methods measure only the effect on acute pain.

MODIFICATIONS OF THE METHOD

Murray et al. (1988) used **mice** instead of rats. They injected 0.020 ml of 5% formalin solution into the subplantar region of the hind paw. Morphine at a dose of 2.1 mg/kg s.c. and pentazocine at a dose of 23.8 mg/kg s.c. were active whereas the cyclooxygenase inhibitor zomepirac was inactive even at a dose of 100 mg/kg s.c.

Hunskaar et al. (1986), Hunskaar and Hole (1987) injected a small amount of formalin (20 µl of 1% so-

lution) under the skin of the dorsal surface of the right hind paw of mice. A biphasisic response with an early (0–5 min) and a late (20–30 min) phase with high licking activity was observed. Central acting analgesics were active in both phases, whereas non-steroidal antiinflammatory drugs and corticosteroids inhibited only the late phase. Acetylsalicylic acid and paracetamol were antinociceptive in both phases.

Shibata et al. (1989) again used lower concentrations of formalin (0.025 ml of 0.5% formalin solution) and also mice instead of rats. They found a characteristic biphasic pain response. Centrally acting drugs such as morphine inhibited both phases, whereas according to their data peripherally acting drugs such as acetylsalicylic acid, oxyphenylbutazone and corticosteroids inhibited only the second phase.

Abbott et al. (1995) used the formalin test for scoring properties of the first and second phases of the pain response in rats.

Abbadie et al. (1997) determined the pattern of c-fos expression in the rat spinal cord to study the two phases of the formalin test.

Clavelou et al. (1989) and Dallel et al. (1995) used a modification of the formalin test for assessing pain and analgesia in the **orofacial region of the rat**. After injection into the upper lip, pain intensity was evaluated by the animal's behavior of rubbing of the injected area. A subcutaneous injection of 0.05 ml of 0.92% formaldehyde solution was made into the upper lip, just lateral to the nose. Following injection, the rat was immediately brought back in a test box equipped with a videocamera for a 45 min observation period. The recording time was divided into 15 blocks of 5 min and a pain score was determined for each block, by measuring the number of seconds that the animals spent rubbing the injected area with the ipsilateral fore- or hindpaw. The animals were sacrificed after the end of the experiment to avoid unnecessary suffering.

Tjølsen et al. (1992) attributed the early phase to C-fibre activation, whereas the late phase appeared to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord.

Alreja et al. (1984) used the formalin test for assessing pain in **monkeys** after volunteering of one of the authors to carry out the same procedure on himself.

Corrêa and Calixto (1993) studied the participation of B_1 and B_2 kinin receptors in the formalin-induced nociceptive response in the **mouse**. Pain response was increased after ACE-inhibition and decreased by bradykinin receptor antagonists.

Herman and Felinska (1979) proposed a rapid test for screening of narcotic analgesics in mice by evaluation of behavioral symptoms after subcutaneous injection of EDTA.

Legat et al. (1994), Dumas et al. (1997) induced hyperalgesia in rats by subplantar injection of collagenase (100 µg in 100 µl saline) and rated the behavioral reactions after treatment with analgesics according to a modified formalin-test.

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H.1.2.11 Experimental neuropathy

PURPOSE AND RATIONALE

Partial injury to somatosensory nerves sometimes causes causalgia in humans. Causalgia is characterized by spontaneous burning pain combined with hyperalgesia and allodynia and usually follows an incomplete peripheral nerve injury. Allodynia, a pain sensation due to normally innocuous stimulation, is a particularly troublesome symptom in patients. Bennet and Xie (1988) described a peripheral neuropathy due to nerve constriction in the rat that produces disorders of pain sensation like those seen in man. This method with slight modifications was used by Davar et al. (1991), Mao et al. (1992), Munger et al. (1992), Yamamoto and Yaksh (1992), Tal and Bennet (1993) and reviewed by Bennett (1993).

PROCEDURE

Anesthesia is induced in male Sprague-Dawley rats by inhalation with halothane 4% and maintained at a concentration of 2–3% as needed. After a local incision, the biceps femoralis of each leg is bluntly dissected at midtigh to expose the sciatic nerve. Each nerve is then mobilized with care taken to avoid undue stretching. Four 4-0 chromic gut sutures are each tied loosely with a square knot around the right sciatic nerve. The left sciatic nerve is only mobilized. Both incisions are closed layer to layer with silk sutures and the rats allowed to recover. During the next days, the animals show a mild eversion of the affected paw and a mild-to-moderate degree of foot drop.

The thermal nociceptive threshold is measured according to the method of Hargreaves et al. (1988), (see H.1.2.3). The rats are placed beneath a clear plastic cage ($10 \times 20 \times 24$ cm) upon an elevated floor of clear glass. A radiant heat source (halogen projector lamp) is placed beneath the glass floor on a movable holder and positioned such that is focuses at the plantar area of one hind paw. The time interval between the application of the light beam and the brisk hind paw withdrawal response is measured to the nearest 01. sec.

The maximum hyperesthesia occurs between 7 and 14 days after nerve ligation. Before intrathecal injection of the drug or vehicle, the hind paws are tested 3 times alternatively with 5-min intervals as the baseline data. The left and right test sequence is carried out at 5, 15, 30, 60 and 90 min after injection.

EVALUATION

The mean ±SEM of the paw withdrawal latency (PWL) is plotted. To analyze the magnitude of hyperesthesia, the difference score (DS) is calculated by subtraction the maximum PWL of the control side (left side) from the maximum PWL of the affected side (right side). Maximum PWL is defined as the PWL which was the maximum during the first 30 min after injection. To analyze the drug effects in hyperesthetic rats, the dose is plotted against the change in DS (post-drug difference score minus pre-drug difference score).

MODIFICATIONS OF THE METHOD

The first animal model of painful neuropathy was reported by Wall et al. (1979a,b).The sciatic nerve of rats or mice was sectioned and either tied or implanted in a polyethylene tube sealed at its far end. Moreover, in one modification also the saphenous nerve was cut, such that the hind paw was completely denervated. This procedure which is known as the neuroma model is believed to replicate the human syndromes seen after amputation (phantom pain) or after nerve transection in an intact limb (anesthesia dolorosa). Within several days, the animals begin to self-mutilate the hindpaw on the side of the nerve transection: a behavior named 'autotomy'.

Seltzer et al. (1990) ligated only one half of the sciatic nerve in rats unilaterally. The withdrawal thresholds to repetitive von Frey hair stimulation at the plantar side were decreased bilaterally as were the withdrawal thresholds to CO_2 laser heat pulses. The contralateral phenomena resemble the 'mirror image' pains in humans with causalgia.

DeLeo et al. (1994) performed cryoneurolysis of the sciatic nerve in the rat using a cryoprobe cooled to –60 °C in a 30/5/30 s freeze-thaw-freeze sequence. Autotomy was observed after 4 to 14 days.

Kim and Chung (1992) described an experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Either both the L_5 and L_6 spinal nerves or the L_5 spinal nerve alone of one side of the rat were tightly ligated.

Zochodne et al. (1994) induced a segmental chronic pain syndrome by lumbar intrathecal NMDA infusion.

Malmberg and Yaksh (1992) reported that hyperalgesia mediated by spinal glutamate or substance P receptor is blocked by spinal cyclooxygenase inhibition.

Sotgiu et al. (1996) performed laminectomy from L1 to S2 in anesthetized rats with sciatic chronic constriction injury. For extracellular recording, two tungsten microelectrodes were positioned under a dissecting microscope on the surface of the spinal cord at L2 and L5–L6 level ipsilaterally to the injured nerve, and were advanced at steps of 2 µm. Neuronal activity was conventionally recorded and then digitized; frequency histograms were constructed by computer programs. The search stimulus for dorsal horn neurons at L2 and L5-6 segments was the electrical stimulation of saphenous and sciatic nerve peripheral territories. Natural stimuli (brushing of the skin) and noxious stimuli (calibrated pinching) were defined. After the responses to saphenous stimuli in the neurons were recorded, a small pad of gel-foam soaked with 0.5 ml lidocaine was placed around the intact epineurium proximally to the ligatures on the sciatic nerve. The saphenous stimulation was repeated during the block and after complete recovery of the preblock baseline activity. In this way, the effect of the local anesthetic on the on the spontaneous activity and on the response to a noxious stimulus could be evaluated.

Idänpään-Heikkilä and Guilbaud (1999) used a rat model of **trigeminal neuropathic pain**, developed by Gregg (1973), Jacquin and Zeigler (1983), Vos and Maciewicz (1991), Vos et al. (1994), Vos and Strassman (1995), where the neuropathy is produced by a chronic constriction injury of the infra-orbital branch of the trigeminal nerve, and studied the effects of various drugs on this purely sensory model of neuropathic pain. The head of the rat, which was anesthetized with sodium pentobarbital 50 mg/kg i.p. and treated with 0.4 mg/kg atropine i.p., was fixed in a stereotaxic frame. A mid-line scalp incision was made exposing skull and nasal bone. To expose the intra-orbital part of the left infra-orbital nerve, the edge of the orbit, formed by the maxillary, frontal, lacrimal and zygomatic bones, was dissected free. To give access to the infra-orbital nerve, the orbital contents were gently deflected with a cotton-tipped wooden rod. The infra-orbital nerve was dissected free at its most rostral extent of the orbital cavity, just caudal to the infra-orbital foramen. Two chromic catgut (5-0) ligatures (2 mm apart) were loosely tied around the infra-orbital nerve. The ligatures reduced the diameter of the nerve by just a noticeable amount and retarded, but did not interrupt the epineural circulation. The scalp incision was closed with silk sutures.

Before the first actual stimulation session, the rats were allowed to adapt to the observation cage and to the testing environment. During this period, the experimenter reached slowly into the cage to touch the walls with a plastic rod, similar to the ones on which the von Frey filaments were mounted.

For mechanical stimulation, a graded series of ten of von Frey filaments with a bending force between 0.217 and 12.5 g was used. The stimuli were applied within the infra-orbital nerve territory, near the center of the vibrissal pad, on the hairy skin surrounding the mystacial vibrissae. The complete series of von Frey hair intensities was presented in an ascending series and either a brisk withdrawal of the head or an attack/escape reaction was considered as the mechanical threshold. Local injection of an local anesthetic (articaine) into the rostral orbital cavity of the lesioned side, into the close proximity of the ligated infra-orbital nerve increased the mechanical threshold to the upper level. The duration of the effect was dose-dependent.

In order to simulate pain experienced by humans in migraine attacks, Storer and Goadsby (1997) developed a model of **craniovascular pain in cats** by stimulating the superior sagittal sinus and monitoring trigeminal neuronal activity using electrophysiological techniques.

Cats were anesthetized with α-chloralose (60 mg/kg i.p.), paralyzed (gallamine 6 mg/kg i.v.) and ventilated. The superior sagittal sinus was accessed and isolated for electrical stimulation by a mid-line circular craniotomy. The region of the dorsal surface of C_2 spinal cord was exposed by a laminectomy and an electrode placed for recording evoked activity from sinus stimulation and spontaneous activity of the same cells. Signals were amplified and monitored on-line. Cells were recorded that were activated by stimulation of the sinus and were also spontaneously activated. Cells fired with latencies consistent with Aδ and C fibres, generally firing three or four times per stimulus (0.3 Hz, 250 µs duration, 100 V) delivered to the sinus. Both evoked and spontaneous firing could be inhibited iontophoresis of serotonin $(5\text{-HT})_{1B/1D}$ agonists.

Mice that lack protein kinase C gamma (PKCγ) displayed normal response to acute pain stimuli, but they almost completely failed to develop a neuropathic pain syndrome after partial sciatic nerve section, and the neurochemical changes that occurred in the spinal cord after nerve injury were blunted (Malmberg et al. 1997).

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H.1.3 Side effects of central analgesic drugs

H.1.3.1 General considerations

Morphine as the prototype of a central analgesic drug has characteristic side effects relevant to the treatment of patients. These are:

- Respiratory depression,
- Decrease of body temperature,
- Tolerance,
- Physical dependence,
- Abuse liability.

Since the ratio between therapeutic effect and side effects is already different between morphine and codeine, both natural alkaloids from opium, great effort has been made to synthesize compounds with a better ratio of analgesic activity versus side effects. Therefore, methods had to be developed to quantify these side effects in animal pharmacology. Moreover, the detection of a multitude of distinct types of receptors that can interact with opioid drugs or endogenous peptides (e.g. μ, κ, and δ receptor) allows a more selective classification of agonists, antagonists, or agonist-antagonists.

H.1.3.2 Test for respiratory depression

PURPOSE AND RATIONALE

Respiratory depression is one of the most troublesome side effects of opioids. Recent studies suggest that respiratory depression is mediated through μ_2 receptors. The frequency of breathing and the inspiratory volume can be affected differently and have to be measured.

PROCEDURE
Male or female rabbits with a body weight between 2.5 and 3 kg are placed in restraining cages. A mask is placed over the nose of the animals which is connected with a valve opening on exhaling and closing on inhaling. The frequency of breathing is recorded from the opening and closing of the valve. The inspiratory volume is measured with a gasometer. Intravenous injection of morphine hydrochloride in doses between 1 and 10 mg/kg results in a dose-dependent decrease respiratory frequency and respiratory volume. The doses are increased logarithmically after the effect of the preceding dose has subsided.

EVALUATION
Three animals are used for the test compound and the standard. Dose-response curves of the effect on respiratory frequency and volume are compared.

MODIFICATIONS OF THE METHOD
Nelson and Elliott (1967) compared the effects of morphine, morphinone and thebaine on respiration and oxygen consumption in rats.

Ling et al. (1983, 1985) measured blood gas values (pO_2, pCO_2, pH) in unrestrained rats via an arterial cannula. pO_2 and pH decreased whereas pCO_2 increased after morphine.

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H.1.3.3 Decrease of body temperature

In rats a single injection of morphine produces a significant fall in body temperature which is more prominent in restrained than in freely moving animals. Since this side effect of opioid drugs is shared by several other compounds with no central analgesic activity, the test can not be regarded as specific.

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H.1.3.4 Methods for the study of tolerance

PURPOSE AND RATIONALE
The radiant heat or the hot plate method for testing analgesic activity in mice is adopted to measure drug induced changes in the sensitivity to a noxious stimulus.

PROCEDURE
Male mice with an initial weight of 18–20 g are used. They are placed in restraining cages. A painful stimulus is produced by an intense light beam directed to the proximal part of the tail. The subject quickly responds to this stimulus by flicking its tail. The reaction time, the interval between stimulus onset and response, is automatically measured in $\frac{1}{10}$-s increments. Prior to drug administration, 2 control readings of reaction time are measured for each animal. After administration of the drug the test is repeated 15, 30, and 60 min after subcutaneous injection or 30, 60 and 120 min after oral administration. In this way, time of peak activity can be found. Mice showing a reaction time of the average control value plus 2 times the standard deviation in the control experiment are regarded as positive. Running dose-response experiments, ED_{50} values are calculated. Subsequently, the animals are treated for 5 days once every day with a dose which is 4 times higher than the ED_{50} in the first experiment. On the following day, dose-response curves are determined using at least 3 doses. The ED_{50} is calculated again.

EVALUATION
The increase of the ED_{50} values before and after subchronic treatment indicates the phenomenon of tolerance.

CRITICAL ASSESSMENT OF THE TEST
The phenomenon of tolerance is observed not only with opioid analgesics but with many other drugs such as barbiturates, benzodiazepines or ethanol. The measurement of analgesia after single and repeated administration, therefore, has to be regarded as a primary test. Demonstration that a new analgesic does not show a decrease of effectiveness after subchronic treatment with high doses indicates that it is free of the need to escalate dosage to maintain efficacy and represents the first step for establishing the absence of tolerance liability.

MODIFICATIONS OF THE METHOD

Other authors (e.g. Glassman 1971) injected the dose which induced complete analgesia in mice twice daily for a period of 21 days and evaluated the stepwise decay of effectiveness. After 21 days, the effect of 10 mg/kg morphine or 30 mg/kg meperidine i.p. decreased to approximately 50% of the value of the first day.

Langerman et al. (1995) evaluated the acute tolerance to continuous morphine infusion up to 8 h in the rat with various doses using the hot plate and the tail flick assay. Tolerance was observed with the hot plate assay but not with the tail flick assay indicating a supraspinal level of tolerance development.

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H.1.3.5 Tests for physical dependence

PURPOSE AND RATIONALE

Abstinence phenomena similar to those in man after abrupt cessation of chronic treatment with opioid analgesics have been reported in rats and monkeys. On this basis, tests for drug dependence have been developed for monkeys (Seevers and Deneau 1963), for rats (Buckett 1964), and for mice (Way et al. 1969; Von Voigtlander and Lewis 1983).

Villarreal et al. (1977), Rodríguez at al (1978), Collier et al. (1979), Cruz et al. (1991) tested opiate abstinence responses in the guinea pig ileum made dependent *in vitro*.

Opiate abstinence responses in the guinea pig ileum made dependent *in vitro*

A 40-cm long segment of the small intestine of male guinea pigs weighing 600–900 g is removed and placed in a low-magnesium Krebs solution. The terminal section of the guinea pig ileum is used after discarding the portion of 10 cm closest to the ileo-caecal junction. The ileum is cut in eight 3-cm long segments. The intestinal content is gently removed with the aid of an glass rod. To produce opiate dependence, segments are incubated in 500 ml Erlenmeyer flasks containing 480 nM morphine in 250 ml Krebs solution saturated with a 95% O_2/5% CO_2 gas mixture at a temperature ranging between 4° and 6 °C for 1 to 48 h. One h before completion of the incubation time, the segments are removed, placed in glass chambers with 50 ml Krebs solution bubbled with 95% O_2/5% CO_2 gas mixture at 36 °C, mounted on a vertical nickrome electrode with one edge fixed to the chamber plug and the opposite fixed to an isometrical force transducer (Grass FT 03) connected to a polygraph for recording the contractile activity of the longitudinal muscle. The ilea are set up with an initial tension of 1 g and left for a period of 30 min for stabilization. Thereafter, all segments are electrically stimulated with supramaximal rectangular pulses (10–40 V) of 0.5-ms duration at a frequency of 0.1 H.

The response to naloxone is recorded by administration up to 100 nM after morphine exposition is completed. Five min before naloxone administration, the electrical stimulation is suspended. The response to the antagonist is recorded for 20 min and thereafter the electrical stimulation is reinitiated and maintained for 10 min.

Thirty-five min after naloxone administration, various doses of nicotine are administered.

For comparisons, a concentration-response curve for nicotine (1, 31., 5.6, 10, 31, and 56 μM) is obtained in fresh ilea. Moreover, the concentration-response curve for nicotine is obtained in ilea (1) exposed to 10 nM naloxone for 20 min; (2) exposed to 480 nM of morphine for 1 h; and pretreated 10 min with 3 or 10 nM of naloxone and exposed to 480 nM of morphine for 1 h. concentration-response curves for nicotine are depressed after pretreatment with morphine. This effect is dose-dependent antagonized by naloxone.

A correlation between the response to supramaximal electrical stimulation and the abstinence precipitated with 100 nM naloxone as well as a correlation between abstinence and nicotine response after long-term exposure (12–48 h) with 480 nM morphine.

Test for physical dependence in rats

Male albino rats are administered either morphine or saline intra-abdominally twice daily. The starting dose of morphine is 20 mg/kg followed by 40 mg/kg increments daily until by day 11 the level is 420 mg/kg. Maintenance at 400 mg/kg is continued through day 20. The test compound is similarly administered to groups of 10 rats. The daily increments have to be adjusted to a maximum level that is not lethal for the duration of the experiment.

Physical dependence capacity is measured on days 11 and 17 by injecting all animals with 10 mg/kg nal-

orphine i.p. in the morning. Withdrawal symptoms are recorded during a 30 to 60 min period. A 10 point score is used: writhing, 3; squealing, either spontaneous or provoked by touch, 2; diarrhea, 2; teeth chattering, 1; eyelid ptosis, 1; wet-dog-type shaking 1.

Physical-dependence sustaining potency is evaluated by substituting from day 20 through day 23 the test compound in the morphine-dependent rats and scoring for suppression of the abstinence syndrome.

Test for physical dependence in monkeys

Groups of 3 rhesus monkeys (1.5–2.8 kg body weight) are injected subcutaneously twice daily. Morphine is started at 2 mg/kg and increased by increments of 2 mg/kg until by day 20 each injection is 40 mg/kg. The test compound is similarly administered to groups of 3 monkeys. For the test compound, the daily increments are adjusted to a level that is not lethal for the duration of the experiment. The groups are then maintained at their appropriate dose levels through 112 days. On days 35, 60, and 91, 5 mg/kg nalorphine is injected subcutaneously in the morning. On days 50 and 112 all doses are omitted for 24 h. Precipitation of the abstinence syndrome due to either withdrawal from treatment or nalorphine injection is evaluated as 4 degrees: mild, moderate, severe, very severe.

CRITICAL ASSESSMENT OF THE METHOD

The Rhesus monkey has been used for this purpose by several authors. For opioids, an excellent correlation between humans and rhesus monkeys has been shown. However, some discrepancies have been noted. For example, meperidine shows higher physical dependence in monkeys compared with humans and dogs (Aceto 1990). Monkeys were also used for testing dependence liability of several sedative-hypnotic agents by self-administration. According to recent studies, for drug evaluation drug discrimination tests are preferred to self-administration studies (Woods et al. 1993).

MODIFICATIONS OF THE METHODS

Mouse jumping as a simple screening method to estimate the physical dependence capacity of analgesics has been recommended by Saelens et al. (1971). Mice receive seven intraperitoneal injections over 2 days. The test compound is given at doses increasing in multiples of two until a maximally tolerated dose is reached. Two hours after the last injection the animals receive an i.p. injection of 100 mg/kg naloxone and are placed individually into glass cylinders. The number of jumps is recorded during 10 min.

Yoshimura et al. (1993) studied the physical dependence on morphine induced in **dogs** via the use of mini-osmotic pumps. Naloxone-precipitated withdrawal signs were recorded such as hyperactivity, biting, digging, tremors, nausea, hyperthermia, and increased wakefulness, and by EEG activation in the amygdala and hippocampus, followed by a dissociation of the EEG in the cortex (fast wave) from that in the limbic (slow wave) system, increased heart rate, and raised blood pressure. Withdrawal signs were more severe in animals with mini-osmotic pumps than in those receiving the same dose by syringe injections.

Pierce and Raper (1995) studied the effects of laboratory handling procedures on naloxone-precipitated withdrawal behavior in **morphine-dependent rats**.

Pierce et al. (1996) used slow release emulsion formulations of methadone to induce dependence in rats. Withdrawal was induced following intraperitoneal challenge with either naloxone or saline, and dependence was assessed in terms of the presence/absence of 13 nominated withdrawal behaviors.

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H.1.3.6 Tests for addiction liability

H.1.3.6.1 General considerations

Psychological dependence or addiction has been defined as strong preference of the subject to experience the effects of a drug (Deneau 1964). Methods have been developed to characterize the addiction phenomenon in animals as a model for addiction in man. Brain-stimulation reward has been used for drug-induced euphoria by Kornetsky and Bain (1990) based on the early observations of Olds et al. (1956, Olds 1979) on intracranial self-stimulation. Self-administration methods have been used to study the reinforcing effects of drugs (Deneau et al. 1969; Hoffmeister 1979; Littmann et al. 1979; Woolverton and Schuster 1983; Bozarth 1987; Meisch and Carroll 1987; Weeks and Collins 1987; Yokel 1987; Woolverton and Nader 1990). Furthermore, discrimination methods have investigated the potential for drug abuse (Holtzman 1983; Brady et al. 1987; Colpaert 1987; Overton 1987; Hoffmeister 1988; Holtzman 1990).

H.1.3.6.2 Drug discrimination studies

PURPOSE AND RATIONALE

Several authors used discrimination methods to investigate the potential for drug abuse (Shannon and Holtzmann 1976; Holtzman 1983; Brady et al. 1987; Shannon and Holtzmann 1986; Colpaert 1987; Overton 1987; Hoffmeister 1988; Carboni et al. 1989; Holtzman 1990).

PROCEDURE

Rats are trained to press one of two choice levers to avoid or to escape electric shocks which are delivered intermittently beginning 5 s after the start of the trial. The occurrence of a trial is signaled by illuminating light in the operant chamber. A third (observing) lever is mounted in the wall of the chamber opposite the two choice levers and must be pressed before the choice response is made. This contingency prevents the rat from hovering by the appropriate choice lever; thus, the choice response in each trial is relatively independent of the consequences of choice responses in the preceding trials of the session. The rats are tested in 20-trial sessions. The animals should be trained to discriminate a prototype of the drug of interest. Morphine and fentanyl have served well as training drugs for exploring the discriminative effects of classical opiates and other drugs. In order to speed up the procedure, the dose of the training drug should be the highest one that does not impair behavior. For discrimination training, the animal is placed in the operant chamber and trained to perform the required response. In two-choice procedures, the left and the right choice lever are designated for drug and vehicle, respectively, for half of the animals in a group; the designation of choice lever are reversed for the other half of the animals. Acquisition of the discrimination is a function of training sessions rather than the number of days of training. Training continues until the subject reaches a predetermined performance criterion, such as 85–95% of responses during 10 consecutive sessions prior to the first reinforcement. Thirty–eighty training sessions or 6–12 weeks are needed to train the animal. Once stable discrimination performance has been achieved, tests of generalization to novel drug conditions can be interposed among the training sessions. One or two times a week test sessions are conducted to determine the ability of subjects to generalize stimulus control of behavior from the training drug to another drug. At 2 sessions per week, 2–3 weeks are needed to construct a complete stimulus-generalization (i.e., dose response) curve for one drug in one subject.

EVALUATION

Results of the stimulus-generalization test usually are evaluated with the quantitative or graded method, whereby the amount of responding on the choice lever appropriate for the training drug (standard) is expressed as a percentage of the total number of responses during a test (i.e., drug-appropriate plus vehicle appropriate responses). This percentage is than compared with the percentage of drug-appropriate responses normally engendered by the training drug (standard). The discriminative effects of the test drug substitute for those

of the training drug if the 2 percentages are not significantly different from each other. When stimulus control of behavior transfers from one drug to another, it can be inferred that the test drug produced discriminative effects that are similar to those of the training drug.

CRITICAL ASSESSMENT OF THE METHOD

Drug discrimination procedures display a high degree of pharmacologic specificity. While test drugs that resemble the training drug result in dose-dependent drug-appropriate lever selections, drugs that are pharmacologically dissimilar to the training drug typically cause responding on the choice lever appropriate for the drug vehicle. Drugs classified as morphine-like agonists produced dose-dependent morphine-like discriminative effects, with the expected potency mediated by the μ-opioid receptor.

MODIFICATIONS OF THE METHOD

Drug discrimination studies were also performed in other species including **squirrel monkeys, Rhesus monkeys**, and **pigeons** (Hein et al. 1981; Herling and Woods 1981; Bertalmio et al. 1982, 1987; Dykstra et al. 1987, 1988). Monkeys trained to discriminate injections of the μ-agonists codeine, etorphine, or alfentanil generalize to other μ-agonists. On the other hand, monkeys trained to these μ-agonists do not generalize to non-opioid drugs, to opioid antagonists, or to opioid agonists which produce their behavioral effects in the rhesus monkey through opioid receptors other than the μ-receptor. Conversely, monkeys trained to discriminate ethylketazocine, which is a κ-agonist in the rhesus monkey, generalize to κ-opioid agonists, but not to μ-opioid agonists, opioid antagonists, or nonopioid drugs (Wood et al. 1993). Apparently, drugs that are self-administered by rhesus monkeys are μ-agonists, and these are the drugs that also show abuse liability in humans. Iwamoto and Martin (1988) discuss the question whether or not drug self-administration studies in monkeys, used for a long time as scientific approach, can predict the abuse potential of drugs. There are several examples that drugs that are self-administered by animals, such as procaine and nomifensine, are not abused by humans. One the other hand, animals show little tendency to self-administer drugs abused by humans, such as THC, LSD, or DOM. Consequently, for drug evaluation the drug discrimination tests have to be preferred in comparison to self-administration studies.

Meert et al. (1989) used drug discrimination studies to characterize risperidone as a complete LSD antagonist.

Meert and Janssen (1989), Meert et al. (1990) showed differences between ritanserin and chlordiazepoxide in drug discrimination procedures.

Δ^9-Tetrahydrocannabinol discrimination in rats has been proposed as model for cannabis intoxication in humans (Balster and Prescott 1990)

An attempt was made to measure opiate abstinence responses in the guinea pig ileum made dependent *in vitro* (Cruz et al. 1991).

The drug discrimination method has also been applied to study anxiolytic drugs using pentylenetetrazol at subconvulsive doses (Sherman and Lal 1979, 1980; Sherman et al. 1979; Lal and Sherman 1980).

The conditioned taste aversion procedure has been described as a more rapid alternative to two-lever operant procedures in drug discrimination research (Garcia et al. 1955; van Heest et al. 1992).

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H.1.3.6.3 Place conditioning paradigm

PURPOSE AND RATIONALE

The place conditioning paradigm has proven successful in identifying the neural mechanisms of drug reinforcement (van der Kooy 1987; Hoffman 1989; Self and Stein 1992). Two classes of drugs, opiates and psychomotor stimulants, have received the most attention, and in each case an important role of dopamine neurons in the mesolimbic system has been established. Moreover, both receptor subtypes, D_1 and D_2, appear to be involved. Intact serotonergic transmission seems to be important in morphine place conditioning, but has not been observed with amphetamine place conditioning.

To induce place preference, a food-deprived animal is exposed to a box which consists of two compartments (which differ in floor texture and wall color) often joined by a small tunnel. Food delivery is paired with confinement to one compartment and not to the other. Later, in the absence of the rewarding stimulus, the animal demonstrates a relative increase in the amount of time spent in the environment previously associated with food compared to the neutral environment. Conditioning to drugs involves the differential pairing of drug administration to one compartment and vehicle with the other. Most authors (Amalric et al. 1987; Bals-Kubik et al. 1998, 1990; Iwamoto 1988; Shippenberg and Herz 1987) applied the drugs via intracerebroventricular injections.

PROCEDURE

Male Sprague-Dawley rats weighing 250–300 g are anesthetized with 60 mg/kg i.p. sodium hexobarbital and stereotaxically implanted with 23 gauge guide cannulae aimed at the lateral ventricle ($AP = -0.9$ mm, $L = +1.5$ mm, $DV = 3.5$ mm) (Paxinos and Watson 1982).

Place conditioning commences one week after cannula implantation. The apparatus consists of $30 \times 60 \times 30$ cm wooden shuttle-boxes with a clear Plexiglas front. For conditioning sessions, each box is divided into two equal-sized compartments by means of a sliding wall. One compartment is white with a textured floor, the other black with a smooth floor. For testing, the central wall is raised 12 cm above the floor and a 5×2 cm 'neutral' steel mesh is inserted along the line separating the two compartments.

Conditioning sessions are conducted once a day for 6 days and consist of injecting drug or its vehicle on alternate days. The rats are immediately confined to one compartment of the shuttle-box following drug injection and to the other compartment following vehicle injection. All conditioning sessions last 40 min. Test sessions are carried out one day after the last training session, in the drug-free state. The rats are placed on the 'neutral' platform of the test box and allowed free access to both sides of the box for 15 min. A videocamera with integrated stop-watch is used for data recording. The time spent in a particular place (drug, vehicle or neutral platform) is assessed by visual analysis of the recorded videotape.

The intracerebroventricular injections are given with a 30 gauge injection needle attached to a microsyringe via polyethylene tubing. The drug solutions are administered over a 60 s period and the injection needles are left in place for and additional 30 s to ensure complete delivery of the solution. For antagonism tests, groups of rats are intracerebroventricularly injected with the antagonist or vehicle 10 min before the microinjection of the conditioning drug.

At the end of the experiments, the rats are anesthetized and sacrificed by decapitation. The brains are removed and sectioned in a cryostat to verify the location of the cannulae.

EVALUATION

Condition scores represent the time spent in the drug-paired place minus the time spent in the vehicle-paired place, and are expressed as means ±SE. Dose-response curves are analyzed with a one-way random factorial analysis of variance. The Wilcoxon test, in which the time spent in the drug associated place is compared to that in the vehicle-paired place, is used to determine whether individual doses produce significant conditioning. A one-way analysis of variance (ANOVA) followed by the Student Newman-Keul's test is used to determine the statistical significance of effects of the antagonist pretreatment.

MODIFICATIONS OF THE METHOD

In order to distinguish place **preference** and place **aversion**, place conditioning behaviours are expressed by 'post – pre', which is calculated as: [(post value) – (prevalue)], where post and pre values are the difference in time spent in the preferred and the non-preferred sides in the post-conditioning and pre-conditioning tests, respectively. Positive values are indicative for preference, negative values for aversion (Kitaichi et al. 1996).

In addition to place preference, Mucha and Herz (1985) used **taste preference** conditioning.

Perks and Clifton (1996) used sucrose solution to generate a place preference which was subsequently devalued using a LiCl taste aversion procedure.

Brockwell et al. (1996) described a computerized system for the simultaneous monitoring of place con-

ditioning and locomotor activity in rats consisting of 4 independent conditioning boxes, each equipped with 6 pairs of photosensors connected to an Experiment Controller, an electronic board containing a microprocessor, a programmable timer, and 16 K of RAM used to store both instructions and data.

Steinpreis et al. (1996) investigated place preference in Sprague Dawley rats treated with graded intraperitoneal doses of methadone. Place preference for methadone peaked at 4 mg/kg and aversion was produced at 10 mg/kg.

Using the conditioned place paradigm, Mamoon et al. (1995) assessed the rewarding properties of butorphanol in comparison to morphine after unilateral microinjections into the ventral tegmental area of male Lewis rats.

Gaiardi et al. (1997) assessed rewarding and aversive effects of buprenorphine by place preference and taste aversion conditioning. After subcutaneous doses of 0.025, 0.050 and 0.100 mg/kg, buprenorphine caused a significant increase in the amount of time spent on the conditioned side, but no significant decrease of saccharin consumption. Rewarding and aversive effects did not occur within a similar dose range.

Contarino et al. (1997) found no tolerance to the rewarding properties of morphine if the effect of repeated i.p. injections of morphine in prolonged conditioned place preference trials.

Tsuji et al. (1996) studied the effect of microinjections of GABA-agonists and -antagonists into the ventral tegmental area of Sprague Dawley rats on morphine-induced place preference.

Sufka (1994) recommended the conditioned place preference paradigm as a novel approach for analgesic drug assessment against chronic pain which was induced in rats by unilateral injections of Freund's adjuvant into the hind paw.

Conditioned place avoidance was found after naloxone which was attenuated by clonidine (Kosten 1994).

Besides morphine and opioids, other drugs with known or putative addiction liability were tested in the place conditioning paradigm, e.g., cocaine (Lepore et al. 1995; Suzuki and Misawa 1995; Calcagnetti et al. 1996; Martin-Iverson and Reimer 1996; Martin-Iverson et al. 1997), caffeine (Brockwell et al. 1991; Brockwell and Beninger 1996), cannabinoids (Lepore et al. 1995; Sañudo-Peña et al. 1997), LSD (Parker 1996), methamphetamine (Suzuki and Misawa 1995), amphetamine (Hoffman and Donovan 1995; Turenne et al. 1996), methylphenidate (Gatley et al. 1996), fenfluramine (Davis and Parker 1993), 7-OH-DPAT (Khroyan et al. 1995; Chaperon and Thiébot 1996), gamma-hydroxybutyric acid (Martellotta et al. 1997), propofol (Pain et al. 1997), NMDA receptor antagonists (Steinpreis et al. 1995; Papp et al. 1996).

Furthermore, 5-HT_3 antagonists (Acquas et al. 1990), 5-HT_3 receptor agonists (Higgins et al. 1993), dopamine release inhibitors (Schechter and Meehan 1994), D1 receptor antagonists (Acquas and Di Chiara 1994), D3-preferring agonists (Khoyan et al. 1997), antiemetic agents (Frisch et al. 1995) were studied in the place-conditioning paradigm.

Suzuki et al. (1991, 1993) and del Pozo et al. (1996) studied opioid-induced place preference in **mice**.

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H.2 Peripheral analgesic activity

H.2.0.1 General considerations

Most of the so called peripheral analgesics possess anti-inflammatory properties and in some cases also anti-pyretic activity besides analgesia. For many of them the mode of action has been elucidated as an inhibition of cyclooxygenase in the prostaglandin pathway. Nevertheless, new peripheral analgesics have to be tested not only for their *in vitro* activity on cyclooxygenase but also for their *in vivo* activity.

The most commonly used methods for measuring peripheral analgesic activity are the writhing tests in mice (various modifications) and the RANDALL-SELITTO-test in rats.

H.2.0.2 Writhing tests

PURPOSE AND RATIONALE

Pain is induced by injection of irritants into the peritoneal cavity of mice. The animals react with a characteristic stretching behavior which is called writhing. The test is suitable to detect analgesic activity although some psychoactive agents also show activity. An irritating agent such as phenylquinone or acetic acid is injected intraperitoneally to mice and the stretching reaction is evaluated. The reaction is not specific for the irritant.

PROCEDURE

Mice of either sex with a weight between 20 and 25 g are used. Phenylquinone in a concentration of 0.02% is suspended in a 1% suspension of carboxymethylcellulose. An aliquot of 0.25 ml of this suspension is injected intraperitoneally. Groups of 6 animals are used for controls and treated mice. Preferably, two groups of 6 mice are used as controls. Test animals are administered the drug or the standard at various pretreatment times prior to phenylquinone administration. The mice are placed individually into glass beakers and five min are allowed to elapse. The mice are then observed for a period of ten min and the number of writhes is recorded for each animal. For scoring purposes, a writhe is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb. The formula for computing percent inhibition is: average writhes in the control group minus writhes in the drug group divided by writhes in the control group times 100%. The time period with the greatest percent of inhibition is considered the peak time. A dose range is reserved for interesting compounds or those which inhibit writhing more than 70%. Compounds with less than 70% inhibition are considered to have minimal activity.

EVALUATION

A dose range is run in the same fashion as the time response except 8 animals/ group are tested at the peak time of drug activity. Four drug groups and a vehicle control group are employed. Animals are dosed and tested in a randomized manner. An estimated ED_{50} is calculated. Doses of 1.0 mg/kg p.o. indomethacin, 30 mg/kg p.o. acetylsalicylic acid, 40 mg/kg p.o. amidopyrine and 80 mg/kg p.o. phenacetin have been found to be ED_{50} values.

CRITICAL ASSESSMENT OF THE TEST

In this test both central and peripheral analgesics are detected. The test, therefore, has been used by many investigators and can be recommended as a simple screening method. However, it has to be mentioned that other drugs such as clonidine and haloperidol also show a pronounced activity in this test. Because of the lack of specificity, caution is required in interpreting the results, until other tests have been performed. Nevertheless, a good relationship exists between the potencies of analgesics in writhing assays and their clinical potencies.

MODIFICATIONS OF THE METHOD

Instead of a phenylquinone suspension, 0.1 ml of a 0.6% solution of acetic acid is injected intraperitoneally to mice with an weight between 18 and 25 g (Koster et al. 1959; Taber et al. 1969). The response is similar to that after phenylquinone. Some authors have used this method together with observation of changes in capillary permeability in order to distinguish between narcotic and non-narcotic analgesics (Whittle 1964).

Eckhardt et al. (1958), Collier et al. (1968), Loux et al. (1978) showed that several substances are able to elicit the writhing response. For example, Amanuma et al. (1984) as well as Nolan et al. (1990) used as irritant intraperitoneal injections of acetylcholine.

Emele and Shanaman (1963), Burns et al. (1968) proposed bradykinin being a endogenous transmitter of pain as irritant.

Sancillo et al. (1987) induced abdominal constriction in mice by intraperitoneal injection of 31.6 µg/kg of prostaglandin E_1.

Bhalla and Bhargava (1980) described a method for assessing aspirin-like activity using aconitine to induce writhing.

Adachi (1994) described a device for automatic measurement of writhing in mice.

Analgesic effects of non-acidic non-steroidal anti-inflammatory drugs in the acetic acid writhing test after intracisternal administration have been found by Nakamura et al. (1986).

The writhing phenomenon can also be observed in **rats** (Fukawa et al. 1980). The writhing responses were induced by intraperitoneal injection of 4% sodium chloride solution. Narcotic and non-narcotic analgesics, antipyretic and nonsteroidal anti-inflammatory drugs were effectively evaluated at relatively low doses. Methamphetamine also showed an analgesic action. VonVoigtlander and Lewis (1982, 1983) induced writhing in rats by injection of 7 ml air or 6% aqueous saline into the peritoneal cavity.

Ethacrinic acid-induced writhing response in rats was used by Björkman et al. (1992).

Schweizer et al. (1988) described a photoelectronic motility monitoring apparatus to measure automatically the writhing movements. A good correlation was found between ED_{50} values after oral administration in mice and the clinically effective oral doses in man.

Heapy et al. (1993) induced the abdominal constriction response in mice by intraperitoneally injecting 0.4 ml of either 0.25% acetic acid, 7.5 mg/ml kaolin suspension, 2.4 mg/ml zymosan solution, or 25 µg/ml bradykinin solution.

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H.2.0.3 Pain in inflamed tissue (RANDALL-SELITTO-test)

PURPOSE AND RATIONALE

This method for measuring analgesic activity is based on the principle that inflammation increases the sensi-

tivity to pain and that this sensitivity is susceptible to modification by analgesics. Inflammation decreases the pain reaction threshold and this low pain reaction threshold is readily elevated by non-narcotic analgesics of the salicylate-amidopyrine type as well as by the narcotic analgesics. Brewers yeast has been used as an inducer for inflammation which increases pain after pressure.

PROCEDURE

Groups of male Wistar rats (130 to 175 g) are used. Only for oral testing the animals are starved 18 to 24 h prior to administration. Otherwise, the route of administration can be intraperitoneal or subcutaneous. To induce inflammation, 0.1 ml of a 20% suspension of Brewer's yeast in distilled water is injected subcutaneously into the plantar surface of the left hind paw of the rat. Three hours later, pressure is applied through a tip to the plantar surface of the rat's foot at a constant rate by a special apparatus to the point at which the animal struggles, squeals or attempts to bite. The apparatus being used has been modified by various authors such as using the Analgy Meter (Ugo Basile, Apparatus for Biological Research, Milan, Italy). Each animal is tested for its control pain threshold. Any animal with a control pain threshold greater than 80 g is eliminated and replaced.

For a time response, groups of at least 7 animals are used, four groups for the agent to be tested and one for the vehicle control. The tests are done at 15 min intervals after subcutaneous administration and at 30 min intervals after oral administration for any change in pain threshold. The interval of time which indicates the greatest increase in pain threshold is regarded as the peak time.

A dose range is obtained in the same manner as the time response. The drug to be tested is administered in a randomized manner. The pain threshold is recorded at time zero and again at the determined peak time.

EVALUATION

The mean applied force is determined for each time interval tested. The percentage increase in pain threshold is calculated by subtracting the applied force of the vehicle control from the applied force of the drug group which is divided by the applied force of the vehicle control in order to give the percentage of increase in pain threshold of the drug group. Doses of 50 mg/kg s.c. Na salicylate, 50 mg/kg amidopyrine, 3 mg/ kg s.c. morphine, 12.5 mg/kg s.c. codeine or pethidine have been found to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The method originally described by RANDALL and SELITTO has been used by many investigators and has been proven to detect central analgesics as well as peripheral analgesics. Peripherally acting analgesics such as the nonsteroidal anti-inflammatory drugs increase only the threshold of the inflamed paw, whereas opiate analgesics increase also the threshold of the intact paw (Dubinsky et al. 1987). In most modifications, the assay has a shallow dose-response curve. Nevertheless, the ED_{50} values of nonsteroidal anti-inflammatory drugs in this test showed a good correlation with human doses (Romer 1980).

MODIFICATIONS OF THE METHOD

The test has been modified by various authors. In some instances the pressure on the inflamed paw has been omitted. Instead the animals were allowed to walk on a metal grid. The gait of the animals is assessed by an observer using a scoring system:

- 0 = three-legged gait
- 0.5 = marked limping
- 1 = normal gait.

The scores are transformed into percent analgesia. Other noxious stimuli were used to induce inflammation and hyperalgesia, such as carrageenin (Winter et al. 1962), Freund's adjuvant or prostaglandin E_2 (Ferreira et al. 1978a).

Vinegar et al. (1990) injected 0.1 ml of 0.25% solution of trypsin into the subplantar region and applied the load force 60 min later. They found a biphasic hyperalgesia and relatively low ED_{50} values for central and peripheral analgesics.

Technically, the method has been improved by several authors, such as Takesue et al. (1969).

Chipkin et al. (1983) modified the test by decreasing the rate of acceleration of the noxious stimulus (mechanical pressure) on the inflamed paw from 20 to 12.5 mm Hg/s and an extension of the cut-off time from 15 to 60 s. This modification is claimed to discriminate analgesics active against mild to severe clinical pain (narcotic-like) from those only useful against mild to moderate pain (non-narcotic-like).

Randall-Selitto analgesy meters are commercially available (e.g., IITC Life Science, Woodland Hills, CA, USA).

Central and peripheral analgesic action of aspirin-like drugs has been studied with a modification of the Randall-Selitto method applying constant pressure to the rat's paw by Ferreira et al. (1978b).

A modification of an analgesia meter for paw pressure antinociceptive testing in neonatal rats was described by Kitchen (1984).

Learning and retention has been tested in rats by Greindl and Preat (1976) inducing pain by a light quantifiable pressure applied to the normal hind paw.

Hargreaves et al. (1988) described a sensitive method for measuring thermal nociception in cutaneous hyperalgesia in rats. One paw was injected with 0.1 ml carrageenan solution, the other paw with saline. The rats were placed in chambers with glass floor and radiant heat was directed to the paws. A photoelectric cell detected the light reflected from the paw and turned off the radiant heat when paw movement interrupted the reflected light.

Perkins et al. (1993) described hyperalgesia after injection of 100 µl of Freund's adjuvant into the knee of anesthetized rats. After 64–70 h the animal was placed with each hind paw on a pressure transducer and a downward force was exerted until the uninjected leg was bearing 100 g. At this point animals were less tolerant to a load on the injected leg, indicating a hyperalgesic response.

Davis et al. (1996) induced mechanical hyperalgesia by injection of substance P and capsaicin in the rat knee joint and measured the download force tolerated by the injected leg.

Ferreira et al. (1993a,b) induced hyperalgesia by intraplantar injection in the hindpaw of rats of various agents, e.g., bradykinin, carrageenin, LPS, PGE_2, dopamine, TNFα, IL-1β, IL-6 and IL-8. A constant pressure of 20 mm Hg was applied to the hind paws and discontinued when the rats presented a typical freezing reaction.

Subplantar injection of 0.1 µg of serotonin in the rat results in a brief period (up to 20 min) of increased pain sensitivity to an applied force (hyperalgesia) which precedes a longer period of decreased pain sensitivity (hypoalgesia). Vinegar et al. (1989) used this phenomenon for pharmacologic characterization of the algesic response.

Similarly, a biphasic algesic behavior after subplantar injection of 250 µg of trypsin was described by Vinegar et al. (1990)

Courteix et al. (1994) proposed the Randall-Selitto paw pressure test in rats with streptozocin-induced diabetes as a model of chronic pain with signs of hyperalgesia and allodynia that may reflect signs observed in diabetic humans.

Amann et al. (1955, 1996) evaluated local edema and effects on thermal nociceptive threshold after intraplantar injection of nerve growth factor into the rat hind paw and studied the effect of a 5-lipoxygenase inhibitor in this test.

Zhou et al. (1996) tested the effects of peripheral administration of NMDA, AMPA or KA on pain behavior in rats. A 28-gauge needle was inserted in the skin of rats proximal to the footpads and advanced about 1 cm so that the tip reached the base of the third toe. A bolus of 20 µl containing concentrations between 1 and 10 000 µM of KA, NMDA or AMPA. For behavioral testing, each animal was placed in a Plexiglas box on a wire mesh screen. Mechanical stimuli were applied using four von Frey filaments with different bending forces. Each von Frey filament was applied 10 times to the skin on the base of the third toe. The paw withdrawal was rated as scores allowing dose-response curves for the hyperalgesic effects of excitatory amino acids. Furthermore, using the highest concentration of the stimulant, effects of antagonists were tested.

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H.2.0.4 Mechanical visceral pain model in the rat

PURPOSE AND RATIONALE

Animal models designed to test the effectiveness of analgesic agents against visceral pain typically rely on noxious chemical irritation of the peritoneum, e.g., acetic acid and phenylquinone induced writhing tests based on acute inflammation. Ethical constraints prevent repeated assessments in a single animal, thereby compounding the difficulty of assessing development of tolerance to analgesic agents. To overcome these constraints, a model for mechanical visceral pain was developed based on repeatable and reversible distension of duodenum in the rat (Coburn et al. 1989; deLeo et al. 1989).

PROCEDURE

A one-piece balloon catheter is prepared composed of PE 50 tubing with a terminal latex rubber balloon which is 7.5 mm long and distensable to hold more than 1.5 ml fluid. Male Sprague Dawley rats weighing 175–200 g are anesthetized with N_2O and halothane. The abdomen is shaved and an 2.5 cm incision is made transversely just below the left costal margin. On the greater curvature of the stomach an incision is made 10 to 20 mm above the pylorus and a purse string is accomplished with 4–0 silk prior to gastrostomy. Through a 2 mm gastrostomy the catheter is introduced and advanced through the pylorus to the first portion of the duodenum (approximately 15–20 mm from the pylorus). The purse string is tied snugly closing the gastrostomy around the catheter. The catheter is tunneled to the base of the scull, externalized and anchored to the dermis with a silicon sleeve and suture. The animals recover from anesthesia within 5 min. Following a 4–5 days recovery period, the duodenal distension volume is determined by the mean threshold that produces writhing (usually 0.5 to 0.7 ml). For the test, the animals are randomized and administered either saline, the standard (0.1, 0.25, 1, and 10 mg/kg indomethacin i.p.) or the test drug in various doses prior to challenging. The animals are placed in a polypropylene box and challenged by inflating the balloon with saline, using a 1 ml calibrated syringe, pulsed 5 times over 30 s and then distended for 1 min. Behavioral responses are scored:

- 0 = Normal behavior defined as exploration, escape attempts and resting
- 1 = Slightly modified behavior defined as cessation of exploration, focusing, wet-dog shake, excessive facial grooming, teeth chattering and deep breathing
- 2 = Mildly to moderately modified behavior defined as retching-like activity, hunching, abdominal grooming or nipping and immobility of the hind limbs (disappears with removal of the stimulus).
- 3 = Severely modified behavior defined as stretching of the hindlimbs, arching and dorsoflection of the hind paws.
- 4 = Intensive visceromotor activity defined as repetitive stretching of the body, extension of the hind limbs, and pelvis, frequent rotating sideward, i.e., writhing.

EVALUATION

The average scores of the groups are plotted on semi log paper and ED_{50} values are determined by best line fit.

CRITICAL ASSESSMENT OF THE METHOD

In the mechanical visceral pain model in the rat, morphine and indomethacin have been found to be active but not other agents involved in prostaglandin inhibition, like acetylsalicylic acid and mefenamic acid. Other mechanisms besides those involving the arachidonic acid cascade have to be investigated.

MODIFICATIONS OF THE METHOD

Ness and Gebhart (1988) used colorectal distension as a noxious visceral stimulus in awake, unanesthetized, unrestrained rats. A 7–8 cm flexible latex balloon was inserted intra-anally under ether anesthesia and kept in position by taping to the base of the tail. Opening a solenoid gate to a constant pressure air reservoir initiated

a 20 s, constant pressure stimulus in the descending colon and rectum. Femoral arterial and venous catheters were tunneled subcutaneously and exteriorized at the back of the neck. Teflon-coated stain-less steel wire electrodes were stitched into the external oblique musculature immediately superior to the inguinal ligament for electromyographic recordings. Blood pressure and heart frequency increase were proportional to the degree of colorectal distension. These effects could be dose-dependently antagonized by morphine and clonidine.

Renal pelvis distension with a pressure of 80 cm H_2O causes a decline in mean arterial blood pressure in pentobarbital-anesthetized rats. Brasch and Zetler (1982) used this blood pressure response, which disappears rapidly after cessation of the distension, to study the effects of analgesic drugs known to be effective in renal colic pain in man.

Moss and Sanger (1990) measured falls in diastolic blood pressure and intragastric pressure after distension of the duodenum by rapid application of intraluminal pressure (10–75 cm H_2O) in anesthetized rats. The distension-induced responses were blocked by pretreatment with morphine, an action reversible by injection of naloxone. Bilateral cervical vagotomy reduced the distension-evoked fall in intragastric pressure but had no effect on the corresponding fall in blood pressure.

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H.2.0.5 Antagonism against local effects of bradykinin

PURPOSE AND RATIONALE

Guzman et al. (1962) and Lim et al. (1964) described the responses (vocalization, respiratory and blood pressure changes) to intra-arterial injection of bradykinin and other algesic agents in cats and dogs. Deffenu et al. (1966) and Blane (1968) used the bradykinin-induced effects after intra-arterial injection in rats as an assay for analgesic drugs. Paravascular sensory nerves which accompany blood vessels throughout the body to end in unmyelinated free-branching terminals close to the capillaries and venules most likely carry the chemoreceptors of pain (Lim 1970). Due to rapid enzymatic degradation, bradykinin is ineffective as noxious stimulus after intravenous or oral administration.

PROCEDURE

Male Wistar rats weighing 280–320 g are lightly anesthetized with ether. A polyethylene catheter with an internal diameter of 0.5 mm is inserted centripetally into the right carotid artery. The catheter is passed through the subcutaneous tissues to protrude from the back of the animal. One hour after recovery from anesthesia, the first dose of bradykinin is injected into the catheter producing dextro-rotation of the head, flexing of the forelimb and occasionally squeaking. For each rat the minimum dose of bradykinin is determined necessary to provoke these effects. The test compounds are applied subcutaneously or intraperitoneally 15 min prior to injection of the threshold dose of bradykinin. The bradykinin injections are repeated in 5 min intervals until the bradykinin effect reappears. Each rat receives one drug at one dose level.

EVALUATION

The criterion for protection is the disappearance of the bradykinin effect after at least 2 consecutive doses of bradykinin. Using groups of 10 rats for various dose levels, ED_{50} values are calculated.

CRITICAL ASSESSMENT OF THE METHOD

Not only narcotic analgesics, but also pyrazolones and phenacetin or acetylsalicylic acid are active in this test. In some animals, the bradykinin-induced response can be diminished after repeated injections, classified as the noxious-adaptable group (Satoh et al. 1979).

MODIFICATIONS OF THE METHOD

Haubrich et al. (1990) tested analgesic activity by the intracarotid bradykinin-induced head/forepaw flexion in the rat. Male Charles River rats (280–320 g) fasted overnight were prepared surgically under light ether anesthesia by insertion of a capped polyethylene cannula (PE-60) centripetally into the right carotid artery and then exteriorizing the cannula to a harness on the back, to permit repeated i.a. injections. The rats were allowed to recover at least 2 h from the surgery, and then given single i.a. injections of bradykinin (triacetate salt in 0.2 ml 0.9% NaCl per injection) at 10-min in-

tervals to determine the threshold dose which produced marked dextrorotation of the head and flexion of the right forepaw of each rat. This response was elicited by threshold doses of bradykinin ranging from 0.1 to 0.5 µg/injection. After administration of the test drugs, the response of the threshold dose of bradykinin was determined at 10-min intervals for 1 h and then at 20-min intervals during the second hour. ED_{50} values were determined by probit analysis of the maximum percentage of rats that failed to respond to bradykinin at each dose of test drug any time within the 2-h test period.

Collier and Lee (1963) described nociceptive responses of **guinea pigs** to intradermal injections of bradykinin and kallidin-10.

Vargaftig (1966) measured the effect of non-narcotic analgesics on the hypotension induced by intra-arterial injection of bradykinin in **rabbits**.

Adachi and Ishii (1979) used the response to injection of bradykinin into the femoral artery of guinea pigs for quantitative assessment of analgesic agents.

Griesbacher and Lembeck (1987) and Lembeck et al. (1991) used the reflex hypotensive response as an indicator of nociception after injection of bradykinin into the ear artery of anesthetized rabbits. Rabbits were anes-thetized and the blood pressure was recorded from the carotid artery. The central artery of one ear was cannulated and the ear was separated from the head with the exception of the auricular nerve, which remained connected to the head. The ear was perfused with Tyrode solution to which acetylcholine and bradykinin were added. The reflex fall in blood pressure induced by bradykinin and acetylcholine were monitored. The effect could be inhibited by bradykinin antagonists.

Heapy et al. (1993) tested the effects of the bradykinin antagonist HOE140 on the abdominal constriction response after intraperitoneal injection of bradykinin to **mice**.

Further methods used to study the role of bradykinin and bradykinin antagonists in inflammation and algesia

Teixeira et al. (1993) investigated the mechanisms of inflammatory response induced by extracts of *Schistosoma mansoni* larvae in guinea pig skin. *Biomphalaria glabrata* snails with patent *Schistosoma mansoni* infections were induced to shed cercariae by exposure to light and water with a temperature of 31 °C. The cercariae were concentrated, homogenized and extracts prepared. Purified eosinophils or neutrophils obtained from peritoneal exudates were radiolabeled by incubation with ^{111}In chelated to 2-mercaptopyridine-*N*-oxide. Radiolabeled leukocyte infiltration and edema formation were measured simultaneously at injected skin sites. [^{125}I]Human serum albumin was added to the labeled leukocytes and these were injected i.v. into anesthetized guinea pigs. After 15 min the extracts of cercariae were locally injected with/or without the inhibitors. After 2 h, the animals were sacrificed and the injected sites were punched out with a 17-mm punch. Serum exudation and leukocyte infiltration were measured by counting the two isotopes. The bradykinin antagonist HOE 140 reduced substantially the extract-induced inflammation.

Ahluwalia et al. (1994) induced plasma protein extravasation in the rat urinary bladder by i.p. injection of cyclophosphamide mediated by capsaicin-sensitive primary afferent neurons which could be significantly inhibited by the bradykinin B_2 receptor antagonist HOE140 and the tachykinin NK_1 receptor antagonist RP67,580.

Davis and Perkins (1994a,b) described a model of persistent inflammatory mechanical hyperalgesia using intra-articular injections of bradykinin or cytokines into the knee joint of rats.

Lecci et al. (1995) analyzed the local and reflex responses to bradykinin on rat urinary bladder motility *in vivo*.

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H.2.0.6 Effect of analgesics on spinal neurons

PURPOSE AND RATIONALE

The mode of action of peripheral analgesics is still a matter of debate. Besides inhibition of the arachidonic acid derived pathway, activities on the spinal and central level have been discussed. Schaible and Schmidt (1983a,b, 1985, 1987, 1988) performed electrophysiological experiments in anesthetized cats and rats after local mechanical stimulation and after induction of acute arthritis of the knee joint. With this method Xe et al. (1990a,b), Neugebauer et al. (1994) found evidence for a spinal antinociceptive action of antipyretic analgesics, such as dipyrone.

PROCEDURE

In cats weighing 2.0–4.0 kg anesthesia is induced by i.m. injection of 15–30 mg/kg ketamine hydrochloride followed by i.v. injection of 60 mg/kg α-chloralose. After immobilization with i.v. pancuronium bromide the cats are artificially ventilated. The skin of the right thigh is incised from rostral of the inguinal fossa to a point below the medial condyle of the tibia. The tendon of the sartorius muscle is cut close to its insertion at the capsule of the knee joint. The muscle is removed to expose the medial aspects of the joint and the medial articular nerve (MAN). The thigh is rigidly fixed to the mounting table by a threaded bolt fitted through the femur so that the lower leg can be flexed and extended in the horizontal plane. The saphenous nerve is cut in the inguinal fossa for recording. Bipolar electrodes are inserted at the MAN near the knee for en passent stimulation of articular afferents. **Extracellular recordings from single MAN units** in the saphenous nerve are performed using platinum wire electrodes. According to their conduction velocities units are classed as group IV afferents (<2.5 m/s, unmyelinated axons) or group III afferents (2.5–20 m/s, thinly myelinated axons).

For **recordings from spinal cord neurons** the spinal segments T12–L7 are exposed by laminectomy. The spinal cord is transected at the lower thoracic region after injection of 0.1 ml of 1% procaine hydrochloride solution to prevent mechanical activation of axons in the long spinal tracts. The animals are fixed to a rigid frame with spinal and pelvic clamps. A pool is formed by skin flaps and filled with warm paraffin oil. The upper lumbar spinal cord is mounted on a pair of platinum wire stimulating electrodes surrounding the whole cord. Ascending tract neurons are identified by electrical stimulation (Neugebauer and Schaible 1990). Single spinal neurons that can be excited by mechanical stimulation of the knee joint tissue are recorded extracellularly using glass-insulated carbon filament electrodes. The neurons are either nociceptive specific neurons responding only to noxious mechanical stimuli or wide dynamic range neurons responding to innocuous stimuli but showing strongest responses to stimuli of noxious intensity.

Acute arthritis in the right knee joint is induced several hours before recordings are started by injecting 0.3–0.5 ml of 4% kaolin suspension and 15–20 min later 0.3 ml of 2% carrageenan solution. Acute arthritis develops within 1–3 h.

Action potentials are displayed on a storage oscilloscope, amplified, filtered, fed to a window discriminator and processed using an interface and a personal computer for construction of peristimulus time histograms. After a control period of at least 40 min during which a stable discharge rate of the afferent or spinal cord unit is obtained, the test substances are administered i.v. in various doses. Effects of the test substance on ongoing and mechanically evoked activity (by movements, pressure stimuli) are determined.

EVALUATION

Ongoing activity is counted every min. The means and standard deviations in 10 min periods are calculated before and after drug application. The values after drug injection are calculated as percentage of control values. To calculate the net effects of the different mechanical stimuli, the number of impulses in the preceding 30 s is subtracted from the total discharges during the stimulus. The responses to at least 4 stimuli before drug application are averaged and set to 100%. The responses to the different mechanical stimuli after drug administration are expressed as a percentage of the controls. Statistical significance is evaluated using the *t*-test for unpaired samples.

MODIFICATIONS OF THE METHOD

Several other electrophysiological methods have been applied to elucidate the mode of action of non-opioid analgesic agents. Carlsson et al. (1986, 1988), Jurna and Brune (1990) recorded the activity from single neurons in the dorsomedial part of the ventral nucleus of the thalamus in rats. Activity was elicited by supramaximal stimulation of nociceptive afferents in the sural nerve. In addition, activity was recorded in ascending axons of the spinal cord.

Chapman and Dickenson (1992) studied the spinal and peripheral roles of bradykinin and prostaglandins in nociceptive processing in the rat by recording C-fibre activity in the dorsal spinal horn after injection of formalin into the center of the respective field of the toe of the hind paw.

Dray et al. (1992) described a preparation of the neonatal rat spinal cord with functionally connected tail maintained *in vitro*. The preparation was placed in a chamber and the spinal-cord and tail were separately superfused with a physiological salt solution. Peripheral nociceptive fibers were activated by superfusion of the tail with bradykinin, capsaicin or by a superfusate heated to 48–50 °C (noxious heat). The activation of peripheral fibres was assessed by measuring the depolarization produced in a spinal ventral root.

Malmberg and Yaksh (1992) described a direct analgesic action of NSAIDs through spinal cyclooxygenase inhibition by blocking the thermal hyperalgesia in rats induced after intrathecal administration of excitatory amino acids or substance P.

A simple technique for intrathecal injections by lumbar puncture in unanesthetized mice was described by Hylden and Wilcox (1980).

Mestre et al. (1994) described a method for performing direct intrathecal injections in rats without introducing a spinal catheter.

Bahar et al. (1984) performed chronic implantations of nylon catheters into the subarachnoid space of Wistar rats and marmosets and tested the effects of local anesthetics.

McQueen et al. (1991) investigated the effects of paracetamol and lysine acetylsalicylate on high-threshold mechano-nociceptors by recording neural activity from the inflamed ankle joint in anesthetized rats with mild adjuvant-induced mono-arthritis.

Yamamoto and Yaksh (1992) studied the effects of excitatory amino acid antagonists administered through chronically implanted lumbar intrathecal catheters on the thermal hyperesthetic state induced by unilateral partial ligation of the sciatic nerve in rats.

Hashimoto and Fukuda (1990) described a spinal cord injury model produced by spinal cord compression in the rat.

Aanonsen and Wilcox (1987) tested effects of spinally administered opioids, phencyclidine and sigma agonists on the action of intrathecally administered NMDA in the tail-flick, hot-plate and biting and scratching nociceptive tests in **mice**.

Brambilla et al. (1996) demonstrated that intrathecal administration of AMPA produced a dose-dependent behavioral syndrome in mice characterized by caudally directed biting, which could be antagonized by peripheral administration of AMPA-receptor and NMDA-receptor antagonists.

Aanonsen and al (1990) tested the effect of iontophoretically applied excitatory amino acid agonists, such as NMDA, AMPA, quisqualate and kainate, on the firing rate of rat spinal neurons after peripheral noxious stimulation.

Cumberbatch et al. (1994) studied the roles of receptors for AMPA in spinal nociceptive and non-nociceptive transmission on dorsal horn wide dynamic range neurones in anesthetized spinalized rats. The effects of systemically administered competitive and non-competitive AMPA antagonists were examined on responses to peripheral noxious heat and non-noxious tap stimuli as well as to iontophoretic AMPA and NMDA.

With this technique, Chizh et al. (1994) studied the effects of intravenous administration of AMPA antagonists to iontophoretically applied excitatory amino acids.

Watkins et al. (1994) induced hyperalgesia in rats by intraperitoneally administered lipopolysaccharides as measured by radiation heat tail flick in rats. Intrathecal catheters were implanted into the subdural space surrounding the spinal cord to test the involvement of excitatory amino acids, substance P, CCK and opioids assessing the effects of antagonists.

Mjellem et al. (1993) produced a behavioural syndrome of caudally directed biting in mice by intrathecal injection of either NMDA or AMPA.

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H.3
Anti-inflammatory activity

H.3.0.1
General considerations

Inflammation was characterized two thousand years ago by Celsus by the four Latin words: Rubor, calor, tumor and dolor. Inflammation has different phases: the first phase is caused by an increase of vascular permeability resulting in exudation of fluid from the blood into the interstitial space, the second one by infiltration of leukocytes from the blood into the tissues and the third one by granuloma formation. Accordingly, anti-inflammatory tests have to be divided into those measuring acute inflammation, subacute inflammation and chronic repair processes. In some cases, the screening is directed to test compounds for local application. Predominantly, however, these studies are aimed

to find new drugs against polyarthritis and other rheumatic diseases. Since the etiology of polyarthritis is considered to be largely immunologically, special tests have been developed to investigate various immunological and allergic factors (see Chapter I).

H.3.1 *In vitro* methods for anti-inflammatory activity

H.3.1.1 General considerations

An array of physiological substances, sometimes called autacoids, are involved in the process of inflammation and repair. These include histamine, serotonin, bradykinin, substance P, and the group of eicosanoids (prostaglandins, thromboxanes and leucotrienes), the platelet-activating factor (PAF) as well as cytokines and lymphokines. Their discovery makes the use of *in vitro* studies possible. The influence of non-steroidal anti-inflammatory agents on the eicosanoid pathway gave rise to numerous studies.

H.3.1.2 ^{3}H-Bradykinin receptor binding

PURPOSE AND RATIONALE

Tissue injury or trauma initiates a cascade of reactions which results in the proteolytic generation of bradykinin and kallidin from high-molecular-weight precursors, kininogens, found in blood and tissue. The rapid enzymatic cleavage of kininogens is accomplished by the kallikreins, a group of proteolytic enzymes which are present in most tissues and body fluids. Bradykinin produces pain by stimulating A and C fibers in the peripheral nerves, participates in the inflammatory reaction and lowers blood pressure by vasodilatation. Since its breakdown occurs via the same enzyme responsible for converting angiotensin I into angiotensin II some of the effects of converting enzyme inhibitors may be due to presence of bradykinin. The ^{3}H-bradykinin receptor binding is used to detect compounds that inhibit binding of ^{3}H-bradykinin in membrane preparations obtained from guinea-pig ileum. Two types of bradykinin receptors (BK_1 and BK_2 receptors) are known (Feres et al. 1992; Bascands et al. 1993; Tropea et al. 1994). The existence of a pulmonary BK_3 receptor has been proposed by Farmer et al. (1989). Evidence was obtained for the existence of three subtypes of B_2 receptors, B_{2a}, B_{2b}, and B_{2c} (Seguin et al. 1992; Seguin and Widdowson 1993).

PROCEDURE

Ileum from guinea pigs is cleaned from its content and cut into pieces of 2 cm length. They are homogenized for 30 s in ice-cold TES buffer, pH 6.8, containing 1 mM 1,10-phenanthroline, in a Potter homogenizer. The homogenates are filtered through 3 layers of gauze and centrifuged twice at 50 000 *g* for 10 min with an intermediate rehomogenization in buffer.

For routine studies the final pellets are resuspended in 40 vol of incubation buffer (25 mM TES buffer, pH 6.8, containing 1 mM 1,10-phenanthroline, 0.1% bovine serum albumin, 140 µg/ml bacitracin, 1 mM dithiothreitol, 0.1 µM captopril). In the competition experiment, 50 µl ^{3}H-bradykinin (one constant concentration of $0.5–2 \times 10^{-9}$ M), 50 µl test compound (6 concentrations, $10^{-5}–10^{-10}$ M) and 150 µl membrane suspension from guinea pig ileum (approx. 6.6 mg wet weight/ml) per sample are incubated in a shaker bath at 25 °C for 90 min.

Saturation experiments are performed with 12 concentrations of ^{3}H-bradykinin ($14.2–0.007 \times 10^{-9}$ M). Total binding is determined in the presence of incubation buffer, non-specific binding is determined in the presence of non-labeled bradykinin (10^{-6} M).

The reaction is stopped by rapid vacuum filtration through glass fibre filters. Thereby the membrane-bound radioactivity is separated from the free one. The retained membrane-bound radioactivity on the filter is measured after addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding of ^{3}H-bradykinin
- non-specific binding in the presence of 10 µM bradykinin
- specific binding = total binding – non-specific binding
- % inhibition: 100 – specific binding as percentage of control value

Compounds are first tested at a single high concentration (10 000 nM) in triplicate. For those showing more than 50% inhibition a displacement curve is constructed using 7 different concentrations of test compound. Binding potency of compounds is expressed either as a relative binding affinity (*RBA*) with respect to the standard compound (bradykinin) which is tested in parallel or as an IC_{50}.

$$RBA = \frac{IC_{50}\ \text{standard compound}}{IC_{50}\ \text{compound}} \times 100\%$$

The dissociation constant (K_i) and the IC_{50} value of the test drug are determined from the competition ex-

periment of ^{3}H-bradykinin versus non-labeled drug by a computer-supported analysis of the binding data (McPherson 1985).

Tests for bradykinin receptor types and subtypes

Bradykinin B_1 receptors have been studied in the isolated rabbit carotid artery (Pruneau and Bélichard 1993) and in the rabbit urinary bladder (Butt et al. 1955).

A potent bradykinin B_1 receptor antagonist has been described by Wirth et al. (1991).

Heterogeneity of B_1 receptors has been suggested by Wirth et al. (1992).

Drummond and Cocks (1995) used rings of bovine left anterior descending coronary artery to study endothelium-dependent relaxations mediated by inducible B_1 and constitutive B_2 kinin receptors.

The production of cyclic GMP via activation of B_1 and B_2 kinin receptors in cultured bovine aortic endothelial cells was described by Wiemer and Wirth (1992).

Pharmacological characterization of bradykinin receptors in canine cultured tracheal smooth muscle cells has been reported by Yang et al. (1995).

Bradykinin B_2 receptors and their antagonists have been studied in human fibroblasts by Alla et al. (1993), with the high affinity radioligand [^{125}I]PIP HOE 140 by Brenner et al. (1993), in guinea pig gall bladder by Falcone et al. (1993), in the smooth muscle of **guinea-pig taenia caeci** by Field et al. (1994), in guinea pig ileum membranes by Graneß and Liebmann (1994), Liebmann et al. (1994a), in isolated blood vessels from different species by Félétou et al. (1994), in endothelial cells by Wirth et al. (1994).

The role of B_1 and B_2 receptors and of nitric oxide in bradykinin-induced relaxation and contraction of isolated rat duodenum was studied by Rhaleb and Carretero (1994).

Campos et al. (1996) investigated the effect of pretreatment with bacterial endotoxin on the bradykinin B_1 and B_2 receptor-induced edema in the rat paw and the interaction of B_1-mediated responses with other inflammatory mediators.

Characterization of kinin receptors by bioassays was described by Gobeil and Regoli (1994). Molecular cloning, functional expression and pharmacological characterization of a human bradykinin B_2 receptor gene was performed by Eggerickx et al. (1992).

Simpson et al. (2000) characterized bradykinin analogues on recombinant human bradykinin B_1 and B_2 receptors using a high throughput functional assay which measures intracellular Ca^{2+} responses.

Bradykinin B_2 receptor subtypes were discussed by Liebmann et al. (1994b) and Regoli et al. (1994).

Evidence for a pulmonary B_3 bradykinin receptor has been given by Farmer et al. (1989).

Bradykinin B_3 receptors have been described by Field et al. (1992) in the smooth muscle of the guinea-pig *taenia caeci* and trachea.

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H.3.1.3 Substance P and the tachykinin family

H.3.1.3.1 General considerations

Substance P belongs to the tachykinin family of peptides that share a common carboxy-terminal sequence (Phe-X-Gly-Leu-Met-NH_2). It was first described by von Euler and Gaddum (1931) as a brain and gut extract that stimulates smooth muscle contraction. Bioassay extracts from spinal dorsal roots implicated substance P as a pain neurotransmitter (Lembeck 1953; Lembeck and Holzer 1979). After determination of the amino acid sequence (Chang et al. 1971) the distribution of substance P in the CNS could be studied (Hökfelt et al. 1975). Neurokinins belong like substance P to a group of neuropeptides named tachykinins. Following the discovery of neurokinin A and neurokinin B, three distinct G protein-coupled receptors, NK_1, NK_2 and NK_3, were described (Maggi et al. 1993; Mussap et al. 1993; Patacchini and Maggi 1995).

Neurokinin A and substance P are preferred agonists of the tachykinin NK_1 and NK_2 receptors, whereas neurokinin B preferentially interacts with the tachykinin NK_3 receptor. The receptor sensitivity of these peptides is relatively poor, and it is possible that their actions could be mediated by interactions with their less preferred receptors.

Nomenclature of tachykinins and tachykinin receptors has been discussed repeatedly (Henry 1987; Maggi 2000).

Tachykinin NK_1 antagonists are potent antiemetics, however other possible therapeutic uses, including rheumatoid arthritis, asthma, migraine, pain and psychiatric disorders, were suggested (Longmore et al. 1995). The P-preferring NK_1 receptor has attracted most interest as a CNS target because it is the predomi-nant tachykinin receptor expressed in the human brain, while NK_2 and NK_3 receptor expression is in extremely low abundance or absent. Several NK_1 receptor agonists antagonists were synthesized and evaluated (Snider et al. 1991; Emonds-Alt et al. 1993; Cascieri et al. 1992; Sakurada et al. 1993; Bristow and Young 1994; Jung et al. 1994; Rupniak and Williams 1994; Smith et al. 1994; Vassout et al. 1994; Patacchini and Maggi 1995; Bonnet et al. 1996; Chapman et al. 1996; Herbert and Bernat 1996; Palframan et al. 1996; Ren et al. 1996).

Moreover, agonists and antagonists at the NK_2 receptor (Hagan et al. 1991, 1993; Beresford et al. 1995; Robineau et al. 1995; Kudlacz et al. 1997; Lecci et al. 1997) and at the NK_3 receptor (Guard et al. 1990; Edmonds-Alt et al. 1995; Patacchini et al. 1995; Nguyen-Le et al. 1996; Beaujouan et al. 1997; Sarau et al. 1997) were reported (Longmore et al. 1995; Rupniak 1999).

Understanding the role of substance P in the brain has been complicated by marked species differences in the distribution of the tachykinin receptor types. There appears to be a relative increase in NK_1 receptor density during evolution, such that NK_3 receptors ara abundant in lower vertebrates and mammals but, like NK_2 receptors, are apparently absent in human brain. Preclinical studies with substance P receptor antagonists have been hindered not only by phylogenetic differences in tachykinin receptor expression, but also by pharmacological heterogeneity of the NK_1 receptor and the NK_2 receptor. An other confounding feature of neurokinin receptor antagonist is the blockade of Na^+ and Ca^{2+} channels at high doses which produces effects in various assays that are independent of receptor antagonism (Patacchini and Maggi 1995; Rupniak 1999). Most developments were guided by the effects of substance P as a pain neurotransmitter. Surprisingly, most clinical studies of analgesic activity of NK_1 receptor antagonists were negative (Rupniak and Kramer 1999). However, clinical findings indicated that substance P receptor antagonists ara able to alleviate depression and anxiety in patients suffering from major depressive disorder (Kramer et al. 1998).

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H.3.1.3.2 ^{3}H-Substance P receptor binding

PURPOSE AND RATIONALE

Substance P is an undekapeptide which is widely distributed in the central and peripheral nervous systems and functions as a neurotransmitter/neuromodulator in a variety of physiological processes. Substance P is released from neurons in the midbrain in response to stress where it facilitates dopaminergic neurotransmission and from sensory neurons in the spinal cord in the response to noxious stimuli, where it excites dorsal neurons. In the periphery, release of substance P from sensory neurons causes vasodilatation and plasma extravasation, suggesting a role in neurogenic inflammation. Selective antagonists to substance P found in receptor binding studies may elucidate the physiological role of substance P and may be candidates for anti-inflammatory and analgesic drugs.

PROCEDURE

Fresh porcine brains are obtained from the slaughterhouse. Striata are dissected and homogenized (Ultraturrax®) in 50 mM ice-cold Tris-HCl buffer, pH 7.4) containing 150 mM NaCl, 150 mM KCl, 12 mM EDTA, 200 µM phenylmethylsulfonylfluoride, 40 µg/ml bacitracin, 4 µg/ml leupeptin, and 2 µg/ml chymostatin. These homogenates are then incubated for 30 min at 4 °C before being centrifuged at 30 000 *g* for 20 min at 4 °C and washed twice with 50 mM Tris-HCl (pH 7.4) buffer. Pellets are resuspended in 0.32 M sucrose containing 200 µM phenyl-methyl sulfonylfluoride and 40 µg/ml bacitracin before storage at –80 °C until use.

Sixty-minute incubations are carried out at room temperature in 50 mM Tris-HCl buffer, pH 7.4, containing various concentrations of [^{3}H]substance P ([^{3}H]SP) (0.05–20 nM), 5 mM $MgSO_4$, 40 mg/ml bacitracin, 4 mg/ml leupeptin, and 2 mg/ml chymostatin in the presence of 0.8–1 mg of membrane protein in a final volume of 1 ml. Total binding and nonspecific binding are determined in triplicate in the absence or presence of 1 mM unlabeled substance P. Incubations are terminated by adding 4 ml of ice-cold Tris-HCl buffer (pH 7.4) and membranes are filtered on Whatman glass fiber filters that are presoaked in 0.5% polyethylenimine for a minimum of 3 h to reduce absorption. Filters are then washed three times (5 ml each) using ice-cold Tris-HCl buffer (pH 7.4). Bound radioactivities are determined using a liquid scintillation counter.

EVALUATION

Saturation and competition data are analyzed using a computer program as described by McPherson (1985).

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H.3.1.3.3 Neurokinin receptor binding

PURPOSE AND RATIONALE

The actions of tachykinins are mediated through three subtypes of neurokinin receptors belonging to the G protein-linked receptor family, namely, NK_1, NK_2 and NK_3. Substance P displays highest affinity to NK_1 receptors, whereas neurokinin A and neurokinin B preferably bind to NK_2 and NK_3 receptors, respectively. NK_1 receptors are expressed in a wide variety of peripheral tissues and in the CNS. NK_2 receptors are expressed primarily in the periphery, while NK_3 receptors are primarily expressed in the CNS.

PROCEDURE

Tachykinin NK_1 receptor binding assay is performed in intact Chinese hamster ovary (CHO) cells expressing the human tachykinin NK_1 receptor (Cascieri et al. 1992). The receptor is expressed at a level of 3×10^5 receptors per cell. Cells are grown in a monolayer culture, detached from the plate with enzyme-free cell dissociation solution (Specialty Media), and washed prior to use in the assay. ^{125}I[Tyr^8]substance P (0.1 nM, 2 000 Ci/mmol; New England Nuclear) is incubated in the presence or absence of test compounds (dissolved on 5 µl DMSO) with 5×10^4 CHO cells. Ligand binding is performed in 0.25 ml of 50 mM Tris-HCl, pH 7.5, containing 5 mM $MnCl_2$, 150 mM NaCl, 0.02% bovine serum albumin, 40 µg/ml bacitracin, 0.01 mM phosphoramidon and 4 µg/ml leupeptin. The incubation proceeds at room temperature until equilibrium is achieved (>40 min) and the receptor ligand complex is harvested by filtration over GF/C filters presoaked in 0.1% polyethylenimine using a Tomtek 96-well harvester. Nonspecific binding is determined using excess substance P (1 µM) representing <10% of total binding.

For **NK_2 receptor binding assays** membranes of CHO cells transfected with human ileum NK_2 receptor are used (Hagan et al. 1993; Beresford et al. 1995). The membrane suspensions (5 µg protein) in assay buffer (Tris base (50 mM), $MnCl_2$ (3 mM), bovine serum albumin (0.05%), chymostatin (2 µg/ml) and leupeptin 4 µg/ml, pH 7.4) are incubated for 90 min at room temperature with wash buffer (Tris base (50 mM), $MnCl_2$ (3 mM), lauryl sulphate (0.01%), pH 7.4) or test compound, and [^{3}H]-GR100 679 (0.5 nM final concentration). Nonspecific binding is defined by use of GR159 897 (1 µM).

For *NK_3* **receptor binding assays** guinea pig cortical membranes (Guard et al. 1990) are incubated at room temperature for 60 min with HEPES wash buffer or test compound and [^{3}H]-senktide (final concentration 0.8–1.0 nM). Non-specific binding is defined by addition of eledoisin (10 µM).

EVALUATION

Inhibition curves are analyzed and pIC_{50} values calculated by use of a curve fitting program. pIC_{50} values are converted to inhibition constants (pK_i values) using the Cheng Prussoff equation

$$K_i = IC_{50} / (1 + L / K_D)$$

where L is the ligand concentration and K_D is the dissociation constant. The K_D and B_{max} (maximum number of binding site per mg of tissue) are determined from saturation curves and analyzed by a curve fitting program. Values are expressed as means ±SEM.

MODIFICATIONS OF THE METHOD

Watson et al. (1955) performed substance P binding assays in **ferret brain membranes** and assessed neurokinin NK_1 receptor binding using human lymphoblasts (IM-9 cells).

Rupniak et al. (1997) studied displacement of ^{125}I-[Tyr^8]substance P binding to cloned human tachykinin NK_1 receptors and to ferret brain membranes *in vitro*.

Beattie et al. (1995) used U373 MG cell membranes and cerebral cortical membranes from rat, ferret and gerbil and [^{3}H]substance P for NK_1 receptor binding assays.

Bonnet et al. (1996), McLean et al. (1996) used the IM9 lymphoblastoma cell line expressing the human NK_1 receptor.

Emonds-Alt et al. (1995) studied binding of [^{125}I] Bolton-Hunter labelled substance P to **NK_1 receptors** of rat brain cortex, human lymphoblast cells (IM9), and human astrocytoma cells (U373MG, STTG1), binding of [^{125}I]iodohistidyl-NKA (or [^{125}I]neuropeptide γ) to **NK_2 receptors** of rat or hamster urinary bladder or guinea pig ileum, binding of [^{125}I]iodohistidyl-[Me-Phe^7]NKB (or [^{125}I]Eledoisin) to tachykinin **NK_3 receptors** of rat, guinea pig and gerbil brain cortex., and of [^{125}I]iodohistidyl-[Me-Phe^7]NKB to the human NK_3 re-ceptor, cloned and expressed in CHO cells (Buell et al. 1992).

Cascieri et al. (1992) described the binding of a potent, selective, radioiodinated antagonist to the human neurokinin-1 receptor.

A radioligand of the tachykinin NK_2 receptor was described by Renzetti et al. (1998).

Jordan et al. (1998) evaluated the Cytosensor microphysiometer, a system that measures the extracellular acidification rate as an index of the integrated functional response to receptor activation, as a method to study NK_3 receptor pharmacology and used this system to assess the functional activity of novel compounds at this receptor.

Appell et al. (1998) reported biological characterization of neurokinin antagonists discovered through screening of a combinatorial library. Using stably transfected CHO-K1 cell lines expressing human NK-1, NK-2 and NK-3 receptor subtypes and europium time-resolved fluorescence, primary receptor binding assays were designed to define active compounds. In addition, a secondary, functional assay measuring intracellular calcium flux with the calcium-sensitive fluorophore, fluo-3, in CHO cells transfected with the human NK-1 or NK-2 receptor was used to determine agonist or antagonist activities.

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H.3.1.3.4 Characterization of neurokinin agonists and antagonists by biological assays

PURPOSE AND RATIONALE

Several biological assays have been used to characterize neurokinin agonists and antagonists on their receptors (Review by Regoli et al. 1994).

The following functional assays are recommended for evaluation of antagonists:

For NK_1 receptors

- Inhibition of [Sar^9,$Met(O_2)^{11}$]substance P-induced endothelium-dependent **relaxation of rabbit pulmonary artery**, previously contracted with 0.1 μM noradrenaline (D'Orléans-Juste et al. 1986; Rubino et al. 1992; Emonds-Alt et al. 1993),
- inhibition of [Sar^9,$Met(O_2)^{11}$]substance P or [Sar^9] substance P sulfone-induced contractions of **guinea pig ileum** in the presence of 3 μM atropine and 3 μM mepyramine and indomethacin (Dion et al. 1987; Emonds-Alt et al. 1993; Patacchini et al. 1995; Hosoki et al. 1998; Walpole et al. 1998),
- **rabbit vena cava** stimulated by substance P or [Sar^9, $Met(O_2)^{11}$]substance P (Nantel et al. 1991; Regoli et al. 1994; Gitter et al. 1995; Robineau et al. 1995; Bonnet et al. 1996; Nguyen-Le et al. 1996),
- inhibition of substance P-induced **relaxation of the isolated dog carotid artery** previously contracted with norepinephrine (Snider et al. 1991),
- **rat urinary bladder**, stimulated by the selective agonist [Sar^9,$Met(O_2)^{11}$]substance P and treated with SR 48986 (1.7×10^{-7} mol/l) to eliminate NK-2 functional sites (Rouissi et al. 1993; Nguyen-Le et al. 1996),
- inhibition of **substance P-induced plasma extravasation in the bladder and bronchi** of the guinea pig (Bonnet et al. 1996),
- inhibition of **substance P-induced vasodilation in the nasal mucosa of pigs** using an acoustic rhinometer (Rinder and Lundberg 1996),
- inhibition of **[Sar^9,$Met(O_2)^{11}$]substance P-induced plasma extravasation in guinea-pig bronchi** (Cirillo et al. 1998),
- inhibition of **methacholine-induced contractions of isolated rat tracheal strips** (Tian et al. 1997),
- inhibition of **cyclophosphamide- and radiation-induced damage in the rat and ferret organs** (Alfieri and Gardner 1997, 1998),
- inhibition of **edema formation induced by substance P and antigen in rat skin** (Herbert and Bernat 1996),
- inhibition of **reciprocal hindlimb scratching after in-tracerebroventricular injection of substance P, [Sar^9, $Met(O_2)^{11}$]substance P or septide in mice (Jung et al. 1994) or gerbils** (Smith et al. 1994),
- inhibition of **turning behavior after intracerebroventricular injection of substance P, [Sar^9,$Met(O_2)^{11}$] substance P or septide in mice** (Jung et al. 1994),
- inhibition of **hind paw tapping and chromodacryorrhea after intracerebroventricular injection of ta-chykinin agonists in gerbils** (Graham et al. 1993; Bristow and Young 1994; Rupniak and Williams 1994; Rupniak et al. 1995, 1997; Vassout et al. 1994),

- inhibition of **cis-platin-induced emesis in ferrets** (Rupniak et al. 1997; Singh et al. 1997; Minami et al. 1998).

For NK_2 receptors

- Inhibition of neurokinin A-induced contraction of **isolated rabbit aorta** (Snider et al. 1991),
- inhibition of neurokinin A-induced contraction of **isolated endothelium-deprived rabbit pulmonary artery or hamster trachea** (D'Orléans-Juste et al. 1986; Emonds-Alt et al. 1993; Patacchini et al. 1995),
- the **hamster urinary bladder** (Dion et al. 1987; Maggi et al. 1990; Regoli et al. 1994; Emonds-Alt et al. 1997; Tramontana et al. 1998),
- inhibition of **motor responses induced by intravesival administration of capsaicin** in rats *in vivo* (Lecci et al. 1997),
- **rat esophageal tunica muscularis** (Croci et al. 1995),
- inhibition of **turning behavior induced by intrastriatal injection of Nle^{10}-neurokinin A in mice** (Emonds-Alt et al. 1997).

For NK_3 receptors

- Inhibition of **senktide- or neurokinin B-induced contractions of the rat portal vein** (Mastrangelo et al. 1987; Snider et al. 1991; Emonds-Alt et al. 1993; Patacchini et al. 1995),
- antagonism against **senktide-induced contractions in the isolated rabbit iris sphincter muscle** (Medhurst et al. 1997; Sarau et al. 1997),
- inhibition of **colonic propulsion in rats** (Broccardo et al. 1999)
- inhibition of **turning behavior induced by intrastriatal injection of senktide in gerbils** (Emonds-Alt et al. 1994),
- inhibition of **citric acid-induced cough in guinea pigs** (Daoui et al. 1998).
- The failure of NK_1 receptor antagonists in most clinical tests for analgesia in spite of clear preclinical data is a matter of discussion (Hill R 2000a,b; Urban and Fox 2000).

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H.3.1.4 Assay of polymorphonuclear leukocyte chemotaxis *in vitro*

PURPOSE AND RATIONALE

Leukocyte accumulation is an important aspect of host defense mechanisms. Chemotactic factors attract leukocytes to an infected or inflamed site. The method of Boyden (1962) has been widely employed to measure the chemotactic effects on polymorphonuclear leuko-

cytes. Watanabe et al. (1989) described a rapid assay of polymorphonuclear leukocyte chemotaxis *in vitro*.

PROCEDURE

Two 96-well tissue-culture plates are utilized as one set of multiple Boyden chambers. One plate as multiple lower compartments and the other plate upside down as multiple upper compartments can be tightly sandwiched with the aid of 12 sets of bolts. The holes, into which bolts are set and polymorphonuclear leukocytes (PMN) suspensions are introduced, are opened by a heated stick. Eight sets of 6 holes are made on the bottom of the upper plate to serve as multiple upper compartments for introducing PMN suspensions. Eight sets of polycarbonate filter (approximately 3.2×2.2 cm), cut from a round filter (Nuclepore Co.) with pores of 2 μm in diameter, are sandwiched between the upper and lower plates. One sheet of the filter can separate six sets of the upper compartments from the lower ones. Silicon grease is spread on all the plate surfaces that attach to the filters. The lower compartments are filled with a chemoattractant (400 μl/well) diluted in RPMI-1 640 medium. The eight sheets of filter paper are carefully placed on each set of the lower compartments to avoid air bubbles. The upper plate is positioned over the lower plate and fastened with bolts. The upper compartments are filled with 0.3 ml of PMN suspension (at 10^7 cells/ml). The assembly is incubated at 37 °C for 60 min in a humidified atmosphere containing 5% CO_2. Then the fluid in the upper compartment is decanted and the upper compartments are completely washed with a jet of water. The lower plate is centrifuged at 2 400 rpm for 5 min at room temperature and the supernatant in the well is decanted. The pellet of PMNs is dispersed in 200 μl of phosphate buffered saline containing 0.1% EDTA. Absorbance at 660 nm of each well containing PMN suspension is determined with a 96-well microplate reader. The number of PMNs in the lower compartments is further determined by a Coulter counter.

EVALUATION

The migration rate is calculated as percentage from the number of PMNs in the lower compartment/number of PMNs applied in the upper compartment. The migration rate is dependent on the concentration of the chemoattractant (e.g., zymosan-activated serum). Moreover, a dose dependent decrease of migration rate is achieved by chemotaxis inhibitors.

MODIFICATIONS OF THE METHOD

Nelson et al. (1975) described chemotaxis under agarose as a simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes.

Migration of PMNs in agarose gel was measured after fixation with glutaraldehyde and staining with Giemsa by Shalaby et al. (1987).

Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood were described by Ferrante and Thong (1980).

PMN chemotaxis was measured in multiwell microchemotaxis chambers separated by 5-μm pore size polyvinylpyrrolidone-free polycarbonate membranes by Harvath et al. (1980), Figari et al. (1987).

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H.3.1.5 Polymorphonuclear leukocytes aggregation induced by FMLP

PURPOSE AND RATIONALE

Aggregation of polymorphonuclear leukocytes (PMNs) can be induced by FMLP (formyl-L-methionyl-L-leucyl-L-phenylalanine). The aggregation can be inhibited by xanthine derivatives.

PROCEDURE

PMNs cell suspensions are prepared from peritoneal exudates obtained 17 h after intraperitoneal injection of 10 ml 6% sodium caseinate into Sprague Dawley rats. The cells are washed twice in Geys-balanced-salt-solution (Gibco GBSS) and resuspended to a final concentration of 15×10^6 cells/ml. The test compounds and the standard (pentoxiphylline) are dissolved in GBSS. FMLP (formyl-L-methionyl-L-leucyl-L-phenylalanine) is dissolved in DMSO. The further dilutions are made up to a final concentration of 10^{-7} mol FMLP in GBSS. Before addition of FMLP, the cell suspensions are preincubated for 10 min with the drugs. PMNs aggregation is carried out in a Born aggregometer.

EVALUATION

The results are expressed as change in transmittance, measured in mm on the recorder. The mean peak of the untreated cells is set 100%.

MODIFICATIONS OF THE METHOD

Moqbel et al. (1986) measured the activation of human leukocytes after FMLP in a rosette assay by the change in the expression of complement (C3b) and IgG (Fc) receptors and in a cytotoxic assay by the *in vitro* capacity to adhere to and kill the complement-coated larvae (schistosomula) of Schistosoma mansoni.

Bradford and Rubin (1986) determined the effect of various drugs on IP_3 accumulation evoked by FMLP in neutrophils from New Zealand white rabbits.

Bourgoin et al. (1991) studied the influence of granulocyte-macrophage colony-stimulating factor (GM-CSF) on phosphatidylcholine breakdown by phopsholipase D in human neutrophils.

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H.3.1.6 Constitutive and inducible cellular arachidonic acid metabolism *in vitro*

PURPOSE AND RATIONALE

The various metabolites of arachidonic acid are involved in many inflammatory processes. Arachidonic acid is released from the cellular phospholipid fraction by the action of phospholipase A_2, and subsequently metabolized via two major routes: the cyclooxygenase pathway yielding the primary prostaglandins and thromboxane, and the 5-lipoxygenase pathway yielding the leukotrienes. Thromboxanes, prostaglandins, and leukotrienes play a pathophysiological role in many diseases. Receptor assays for these autocoids were developed (Haluska et al. 1989). Murata et al. (1997) produced mice lacking the prostaglandin receptor which showed increased susceptibility to thrombosis and altered pain reception and inflammatory response.

The therapeutic mode of action of the classical nonsteroidal anti-inflammatory drugs (NSAID), such as aspirin or indomethacin, is primarily explained by their inhibitory effect on cyclooxygenase, the key enzyme of the prostaglandin pathway. Inhibitors of the 5-lipoxygenase pathway have attracted considerable attention as potential anti-inflammatories with high potency. Appropriate assay systems for the determination of the different eicosanoids allow to study the influence of drugs towards the specific pathways of the arachidonic acid cascade in various cellular systems (Samuelsson 1986; Vane and Botting 1987).

According to recent discoveries there are two forms of cyclooxygenase (Xie et al. 1992; Lee et al. 1992; Gierse et al. 1996). Cyclooxygenase-1 (COX-1) is found as a constitutive enzyme in most tissues including blood platelets. Prostaglandins generated by constitutive pathways may exert cytoprotective effects, and are involved in maintaining vital functions in vascular hemostasis, gastric mucosa and kidney.

The inhibition of these prostaglandins by the classical cyclooxygenase inhibitors is now generally accepted as an explanation of their adverse side effects.

COX-2 which shares about 62% amino acid homology with COX-1, is only expressed after cell activation, especially by mitogenic or inflammatory stimuli

(Herrmann et al. 1990; Funk et al. 1991; Crofford 1997). Thus, specific suppression of the COX-2-pathway may represent a superior target for the evaluation of new antiinflammatory drugs. Drugs which have a high potency on COX-2 and a favorable COX-2/COX-1 ratio have potent anti-inflammatory activity with fewer side effects (Riendeau et al. 1997; Vane 1998; Hawkey 1999; Song et al. 1999; Chan et al. 1999).

H.3.1.6.1 Formation of leukotriene B_4 in human white blood cells *in vitro*

PROCEDURE

Human white blood cells are prepared according to the standard procedure published by Salari et al. (1984): 40 ml freshly drawn citrated blood are admixed with 8 ml of PM16-buffer, containing 6% (v/v) dextran (MW = 480 000), and incubated at room temperature for one hour. The supernatant containing the white blood cell fraction is removed, diluted 1 : 1 (v/v) with PM16 and centrifuged for 15 min at 300 *g*. The precipitate is resuspended in PM16 and adjusted to 10^{10} cells/l (Counter HT, Coulter Electronics, Krefeld, FRG).

The metabolism of endogenously bound arachidonic acid to LTB_4 is measured in a total volume of 0.3 ml of the cell suspension at 37 °C. The reaction tube contains 2 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$ and the investigational drug. After 15 min pre-incubation the reaction is started by addition of 12.5 µg of the Ca-ionophore A 23 187 and 2 µg glutathione. After 5 min the reaction is stopped with 30 µl 0.1 M HCl at 0 °C. After centrifugation for 2 min at 0 °C, aliquots of the supernatant are subjected to HPLC, similarly as described by Veenstra et al. (1988), using a C-18 Nucleosil column (5 µm, 100 × 3 mm, Chrompack GmbH, Frankfurt, FRG) and, at a flow rate of 0.7 ml/min, a solvent mixture consisting of 725 ml methanol, 275 ml water and 0.1 ml acetic acid. Authentic standard drugs are used to identify cis-, trans- and epi-LTB_4. The separation of the three isomers can be followed photometrically at the UV-maximum of 278 nm.

EVALUATION

The peak areas of cis-, trans- and epi-LTB_4 are measured as a function of drug concentration, and related to a control experiment without drug.

CRITICAL ASSESSMENT

The tested pathway involves the enzymatic steps of phospholipase A_2 and the 5-lipoxygenase. Thus inhibitors of these enzymes are to decrease formation of the three isomers of LTB_4. The step of phospholipase A_2 can be circumvented by the addition of exogenous arachidonic acid (1 µmol/l).

Inhibitors of LTA_4-hydrolase which catalyzes the intermediary conversion of LTA_4 to the biologically active cis-LTB_4, can also be identified by this assay. Such drugs are to exhibit an increased formation of the non-enzymatic hydrolysis products trans- and epi-LTB_4 at the expense of decreased cis-LTB_4.

MODIFICATIONS OF THE METHOD

Winkler et al. (1988) used differentiated U-937 cells expressing LTB_4 receptors to study Ca^{2+} mobilization in response to LTB_4.

Jones et al. (1995) tested inhibition of [^{3}H]leukotriene D_4 specific binding in guinea pig lung, sheep lung, and dimethylsulfoxide-differentiated U837 cell plasma membrane preparations.

H.3.1.6.2 Formation of lipoxygenase products from ^{14}C-arachidonic acid in human polymorphonuclear neutrophils (PMN) *in vitro*

PROCEDURE

PMN are prepared by the standard procedure of Böyum (1976). The first steps are carried out at room temperature: 50 ml citrated human blood are centrifuged at 130 *g* for 15 min. The pellet is resuspended in 20 ml Dulbecco's minimal essential medium, and subsequently underlayered with 15 ml lymphoprep. After centrifugation at 400 *g* for 25 min the pellet is resuspended in 28 ml Dulbecco's HBSS containing 3% dextran. After incubation at 0 °C for 120 min the decanted supernatant is centrifuged at 400 *g* for 15 min. The pellet is resus-pended in 1 ml Dulbecco's PBS, containing 11 µmol/l glucose, to a leukocyte count of 2×10^{10}/l.

The method of HPLC determination of cellular metabolites of exogenous 1-C-14-arachidonic acid, as published by Borgeat and Samuelsson (1979), is modified as briefly described:

0.1 ml of the leukocyte suspension are incubated in Dulbecco's phosphate buffered saline (DPBS) at 37 °C with the test drugs for 15 min. The incubation is then interrupted by cooling in an ice bath. Calcium ionophore A 23187 (final concentration 7×10^{-5} mol/l) and 1-C-14-arachidonic acid (final concentration 8.4×10^{-5} mol/l, 0.5 µCi) are added, and, after a second incubation period of 15 min at 37 °C, terminated by the addition of 0.4 ml methanol. The assay mixture is then extracted with chloroform. The chloroform is evaporated, and, after redissolving the residue in a minor amount of methanol/water, analyzed by HPLC and radiomonitoring for the C-14-eicosanoids.

HPLC-conditions are as follows: Column: Nucleosil C-18, 5 µm; organic phase: 700 ml methanol, 300 ml water, 0.1 ml acetic acid; after 35 min change to pure methanol. Flow: 1 ml/min, 2 000 psi. Radiomonitor: LB 507 (Berthold, Wildbad, FRG).

A viability assay (trypan blue exclusion) ascertains that cells remain intact during incubation periods.

EVALUATION

The radioactivity of the separated 5-HETE and LTB_4 is measured as a function of drug concentration, and related to a control experiment without drug. Two further lipoxygenase products, 12-HETE and 15-HETE, which are additionally generated under the test conditions, can be quantified in a similar way.

CRITICAL ASSESSMENT

The measured reaction sequence starts with arachidonic acid, and involves its transformation to 5-HETE by 5-lipoxygenase, as well as the subsequent enzymatic hydrolysis to LTB_4. Inhibitors of 5-lipoxygenase exhibit a decreased formation of 5-HETE and LTB_4. Effects of drugs on the side products 12-HETE and 15-HETE can also be studied in this test system.

H.3.1.6.3 Formation of eicosanoids from ^{14}C-arachidonic acid in human platelets *in vitro*

PROCEDURE

Blood is drawn from the vena brachialis of healthy volunteers and collected into plastic tubes containing sodium citrate (0.38% final concentration (w/v)). After centrifugation at 100 *g* for 15 min the platelet rich plasma (PRP) containing about $2{,}5 \times 10^{11}$ platelets/l (Counter HT, Coulter Electronics, Krefeld, FRG) is saved, and kept at 20 °C no longer than one hour until the experiment is started. Metabolism of ^{14}C-arachidonic acid is followed by the HPLC procedures published by Weithmann et al. (1994), modifying the method of Powell (1985). PRP is mixed with the same volume of a citrate/D-glucose solution (27.35 g trisodium citrate · 2 H_2O, 1.47 g citric acid and 27.74 g D(+)-glucose · H_2O ad 1 l water) and centrifuged at 1000 *g* for 20 min (4 °C). The saved precipitate is resuspended in Dulbecco's solution (DPBS, Serva, Heidelberg, FRG) in the original volume. 0.495 ml of this platelet suspension is incubated at 37 °C for 15 min with the test compound. Subsequently eicosanoid formation is started by the addition of 5 µl of 1-^{14}C-arachidonic acid solution (50 Ci/mol, 9×10^{-4} Ci/l, NEN, Dreieich, FRG). After 5 min (37 °C) the reaction is stopped by adding 0.5 ml of chilled acetone/0.1 ml 1n HCl, cooled to 0 °C, and extracted two times with 3 ml ethylacetate. The combined organic extracts are evaporated and the residue redissolved in 0.2 ml methanol. Aliquots are separated by HPLC at a flow rate of 1.5 ml/min, using a C-18 Nucleosil column (5 µm, 125×4.6 mm, Bischoff, Leongang, FRG) connected with a pre-column C-18 Nucleosil (5 µm, 20×4.6 mm) of the same type. The formed ^{14}C-eicosanoids are analyzed using a liquid scintillation flow detector LB 507 (Berthold, Wildbad, FRG). The radiochromatogram is analyzed by comparison with tritiated authentic eicosanoids (NEN, Dreieich, FRG). The elution system consists of the following solvent mixtures (elution time in parenthesis): I 725 ml water/275 ml acetonitrile/1 ml acetic acid (40 min), II 700 ml methanol/300 ml H_2O/1 ml acetic acid (40 min), III pure methanol (20 min)

EVALUATION

The radioactivity of the separated TXB_2 and PGE_2 is measured as a function of drug concentration, and related to a control experiment without drug addition.

A further lipoxygenase product, 12-HETE, which is additionally generated under the test conditions, can be quantified in a similar way.

Lasché and Larson determined PGI_2 by a bioassay based on its generation by aortic rings and assay by its ability to inhibit platelet aggregation.

CRITICAL ASSESSMENT

The measured reaction sequence starts with arachidonic acid, and involves its transformation to prostaglandin endoperoxides, which is catalyzed by cyclooxygenase. The unstable and short-living endoperoxides transform immediately into thromboxane A_2 by the action of thromboxane isomerase. TXA_2 is unstable, too, and yields the stable non-enzymatic hydrolysis product TXB_2.

Inhibitors of cyclooxygenase exhibit a decreased formation of TXB_2. Specific inhibition of the thromboxane isomerase step results in an accumulation of the mentioned endoperoxide intermediates, which due to their chemical instability are non-enzymatically transformed to the primary PGE_2. Thus, inhibitors of the TXA_2-isomerase lead to a significant increase of the PGE_2-peak at the expense of the TXB_2-peak. Effects of drugs on the side product 12-HETE can also be studied in this test system.

H.3.1.6.4 Stimulation of inducible prostaglandin pathway in human PMNL

PROCEDURE

The procedure of Herrmann et al. (1990) with the modification of Weithmann et al. (1994), is used to stimu-

late cyclohexamide-inhibitable generation of PGE_2 in human PMNL by LPS.

H.2.5×10^9 PMNL/l culture medium (RPMI 1 640, completed with 1 mmol/l sodium pyruvate, 5% FCS (w/v), 2 mmol/l glutamine and each 100 U/ml penicillin/streptomycin) are incubated with 100 µmol/l acetylsalicylic acid for 60 min (37 °C, 5% CO_2), and the latter subsequently removed by four times washing with medium. 0.25 ml-aliquots (containing 2.5×10^9 PMNL/l medium) are incubated with 0.1 mg/ml LPS (lipopolysaccharide from Salmonella abortus equii, Sigma GmbH, Deisenhofen, FRG) along with the test compound for 18 h (96-well plates, 37 °C, 5% CO_2). The generation of eicosanoids is induced by the administration of 7×10^{-5} mol/l of the calcium ionophore A 23 187. After incubation for 30 min at 37 °C the plates are centrifuged at 400 *g* for 15 min. PGE_2 is determined in the supernatant, using an ELISA-kit commercially available from several distributors. Alternatively the test compound is added along with the calcium ionophore. At least 90% of the cells remain intact during incubation times (trypan blue exclusion assay).

EVALUATION

The PGE_2-concentration in the sample is determined from appropriate calibration curves. The PGE_2-concentration is measured as a function of drug concentration, and related to a control experiment without drug.

CRITICAL ASSESSMENT

Long term activation with LPS or other inflammatory effectors leads in human polymorphonuclear neutrophils to the stimulation of a prostaglandin synthesizing capacity, which under normal conditions is not present in this system. The described assay system detects compounds which interfere with this activation process.

Incubation of drugs with already activated cells allows to search for drugs that directly influence the enzyme activity.

H.3.1.6.5 COX-1 and COX-2 inhibition

PURPOSE AND RATIONALE

Several assays were described to characterize COX-1 and COX-2-inhibitors, such as *in vitro* COX enzyme assay (Seibert et al. 1994), COX-2 protein extraction and analysis (Anderson et al. 1996), a human whole blood assay using LPS-induced PGE_2 production as an index for cellular COX-2 activity (Riendeau et al. 1997) or whole-cell assays with transfected Chinese hamster ovary cells expressing COX-1 and COX-2 or COX-2 specific (osteosarcoma cells) and COX-1 specific (U937 cells) making use of PGE_2 production after arachidonic challenge as an index of cellular potency and selectivity of cyclooxygenase inhibitors (Chan et al. 1999).

PROCEDURE

Inhibition studies with recombinant human COX-1 and COX-2

Microsomal preparations of recombinant human COX-1 and COX-2 are prepared from a vaccine virus-COS-7 cell expression system (O'Neill et al. 1994). Recombinant human COX-1 and COX-2 are expressed in baculovirus-Sf9 cells, and enzymes are purified (Ouellet and Percival 1995; Cromlish and Kennedy 1996). Enzymatic activity is monitored continuously by either a fluorescence assay measuring the appearance of the oxidized form of the reducing agent cosubstrate homovanillic acid or by oxygen consumption (Ouellet and Percival 1995).

HPLC assay for oxygenation of radiolabelled arachidonic acid by COX-1

Purified recombinant human COX-1 (50 µl of 1 µg/ml in 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenol, 1 µM hematin) is preincubated with 2 µl of the inhibitor solution (50 fold concentrated stock in DMSO, 0–2.5 mM) for 15 min. The reaction is then initiated by the addition of 5 µl of 1 µM [^{14}C]-arachidonic acid (0.005 µCi) to obtain a final concentration of 0.1 µM. After 7 min incubation at room temperature, the reaction is stopped by the addition of 5 µl 1 M HCl and 50 µl acetonitrile. Aliquots of 50 µl of each reaction mixture are analyzed for substrate conversion by reverse phase HPLC onto a C-18 Nova-Pak column (3.9×150 mm) which is developed with acetonitrile/water/acetic acid (85 : 15 : 0.1) at 2 ml/min. Arachidonic acid metabolites and arachidonic acid eluted at 0.6–1 min and 2.2–2.5 min, respectively, are quantitated by a Packard radiochromatography detector. Percentages of inhibition are calculated from the difference in conversion of arachidonic acid to prostaglandin metabolites between inhibitor-treated samples and controls exposed to DMSO vehicle.

Determination of the stoichiometry of inhibitor binding

Aliquots of purified COX-2 (0.25 mg/ml, concentration of subunit of 3.4 mM) are incubated in buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenol) in the presence of various inhibitors (0–8 µM) for 15 or 30 min. An aliquot (20 µl) is then removed for determination of the cyclo-oxygenase activity which is monitored continuously by oxygen consumption by a Clark-type polarographic oxygen probe. The oxygen chamber is filled with 0.6 ml of reaction buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 µM hematin,1 mM

phenol, 100 μM arachidonic acid at 30° or 37 °C) and the reaction is initiated by the addition of 20 μl of a solution of 4 mM hydrogen peroxide and 0.5 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) in assay buffer. Enzyme concentration is determined by amino acid concentration following acid hydrolysis.

Determination of the dissociation rate constant of the enzyme-inhibitor complex

Purified COX-2 (2.0 nmol, 2 ml) in 20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 2 μM hematin, 0.1% β-octyl-glucoside is treated with 2.0 nmol [^{14}C]-DFU (18 Ci/mol) and incubated at 20 °C for 3 h. A control (0.7 ml) is removed and 13 nmol unlabelled DFU is added to the remaining 1.3 ml of the mixture containing COX-2 and [^{14}C]-DFU. At timed intervals, 0.1 ml (in duplicate) is transferred to a Microcon-30 micro concentration device (Amicon) and the free inhibitor is separated from enzyme-bound inhibitor by centrifugation at 14 000 *g* for 6 min at 4 °C. Buffer (0.1 ml) is added to the retentate and the centrifugation repeated. The filtrate and retentate are then removed and mixed with 10 ml scintillation fluid and counted in a liquid scintillation counter.

An aliquot of purified COX-2 (1.0 nmol) is treated with 1.25 mol equivalents of inhibitor or with DMSO vehicle control and incubated at 20 °C for 1 h. The enzyme-inhibitor mixture is then transferred to a Pierce Microdialyzer 100 apparatus and dialyzed continuously against 2 l of buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM phenol, 0.1% octylglucoside) for 5 h at 22 °C during which aliquots are moved and frozen at −70 °C until assayed for cyclyo-oxygenase activity by oxygen uptake as described above.

Recovery of inhibitor from the COX-2-inhibitor complex

Purified COX-2 (0.79 nmol) is treated with 1.0 mol equivalent of inhibitor and the mixture is incubated for 60 min at room temperature. The remaining activity at this time is 4% that of a vehicle-treated control. The sample is then divided in two and the protein denaturated by treatment with four volumes of ethyl acetate/methanol/1 M citric acid (30 : 4 : 1). After extraction and centrifugation (10 000 *g* for 5 min), the organic layer is removed and the extraction repeated. The two organic layers are combined and dried under N_2. The extract is dissolved in 10 μl of HPLC solvent mixture consisting of water/acetonitrile/acetic acid (50 : 41 : 0.1) and 50 μl are injected onto a Novapak C-18 column (3.9 × 150 mm) and developed at 1 ml/min. The inhibitor is detected by absorption at 260 nm and eluted with a retention time of 6.6 min in this system. Control experiments for inhibitor recovery are performed with incubation of the inhibitor in the absence of enzyme and processing of the samples in an identical fashion before quantitation by HPLC.

Spectrophotometric assay of recombinant human COX-2

Enzymatic activity of the purified COX-2 is measured using a chromogenic assay based on the oxidation of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) during the reduction of PGG_2 to PGH_2 (Copeland et al. 1994). The assay mixture (180 μl) contains 100 mM sodium phosphate, pH 6.5, 1 μM hematin, 1 mg/ml gelatin, 2 to 5 μg/ml of purified COX-2, and 4 μl of the test compound in DMSO. The assay is also performed in the presence of the detergent Genapol X-100 at a final concentration of 2 mM. The mixture is preincubated at room temperature (22 °C) for 15 min before the initiation of the enzymatic reaction by the addition of 20 μl of a solution of 1 mM arachidonic acid and 1 mM TMPD in assay buffer (without enzyme or hema-tin). For assays in the presence of Genapol, the arachidonic acid and TMPD solution is prepared in 50% aqueous ethanol. The enzyme activity is measured by estimation of the initial velocity of TMPD oxidation over the first 36 s of the reaction as followed from the increase in absorbancy at 610 nm. A low rate of non-enzymatic oxidation is observed in the absence of COX-2 and is subtracted before the calculation of the percentage of inhibition.

Whole-cell assays with transfected Chinese hamster (CHO) cells expressing COX-1 and COX-2

Stably transfected CHO cells expressing human COX-1 and COX-2 are cultured and assayed for the production of PGE2 after stimulation with arachidonic acid (Kargman et al. 1996). Cells (0.3×10^6 cells in 200 μl) are pre-incubated in HBSS containing 15 mM HEPES, pH 7.4, with 3 μl of the test drug or DMSO vehicle for 15 min at 37 °C before challenge with arachidonic acid. Cells are challenged for 15 min with an arachidonic acid solution (10% ethanol in HBSS) to yield final concentrations of 10 μM arachidonic acid in the CHO[COX-2] assay and 0.5 μM arachidonic acid in the CHO[COX-1] assay. In the absence of addition of exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-1] are <30 pg $PGE_2/10^6$ cells. In the presence of 0.5 μM exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-1] cells increase to 260 to 1 500 pg $PGE_2/10^6$ cells. After stimulation with 10 μM exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-2] cells increase from <120 to 700 to 1 600 pg $PGE_2/10^6$ cells. Compounds are tested in eight concentrations in duplicate using 3-fold serial dilutions in DMSO. COX activity in the absence of test compounds is determined as the difference in PGE_2 levels of cells challenged with arachidonic acid versus PGE_2 levels in cells mock-challenged with ethanol vehicle.

Whole-cell assays with osteosarcoma cells (COX-2) and U937 cells (COX-1)

The human osteosarcoma cell line has been shown to selectively express COX-2 by reverse transcription-polymerase chain reaction and immunoblot analysis, whereas undifferentiated human lymphoma U937 cells selectively express COX-1. The production of PGE_2 by these cells after arachidonic acid challenge is used as an index of cellular COX-2 and COX-1 activity, respectively. Test substances are preincubated for 5 to 15 min with the cells under serum-free conditions (HBSS) before a 10-min stimulation with 10 µM arachidonic acid and measurement of PGE_2 production (Wong et al. 1997). COX activity in each cell line is defined as the difference in PGE_2 concentrations in samples incubated in the presence or absence of arachidonic acid.

Human whole blood assay

For the COX-2 assay, fresh heparinized human whole blood is incubated with lipopolysaccharide from *E. coli* at 100 µg/ml and with 2 µl of vehicle or a test compound for 24 h at 37 °C (Brideau et al. 1996). PGE_2 levels in the plasma are measured using radioimmunoassay after deproteination. For the COX-1 assay, an aliquot of fresh blood is mixed with either DMSO or test compound and is allowed to clot for 1 h at 37 °C. TBX_2 levels in the serum are measured using an enzyme immunoassay after deproteination.

MODIFICATIONS OF THE METHOD

Berg et al. (1997) developed a cell assay system using the human erythroleukemic cell line HEL as a source for COX-1 and the human monocytic cell line Mono Mac 6 as a source for COX-2.

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H.3.1.7 Induced release of cytokines (Interleukin-1alpha, IL-1beta, IL-6, IL-8 and TNF-alpha) from human white blood cells *in vitro*

PURPOSE AND RATIONALE

Cytokines represent a class of different, biologically highly potent peptides, that are endogenously synthesized upon stimulation. They are involved in numerous cellular processes, such as inflammation, immunological responses and many others. Their broad pleiotrophic biological activities are best characterized by their former classification as

- B-cell activating and differentiating factor, endogenous pyrogen, osteoclast activating factor, thymocyte proliferation factor, monocyte cell factor, leukocyte endogenous factor (IL-1);
- hepatocyte stimulatory factor, hybridoma growth factor, haematopoietic cell stimulatory factor (IL-6);
- neutrophil chemotactic factor and adhesion inhibitor (IL-8);
- tumor necrosis factor (TNFalpha).

There are presently 18 cytokines with the name interleukin (IL) (Dinarello 2000). The concept of proinflammatory cytokines and antiinflammatory cytokines (IL-4,IL-10,IL-13) is fundamental to cytokine biology and novel drug discovery strategies.

Blocking IL-1 or TNF has been highly successful in patients with rheumatoid arthritis, inflammatory bowel disease, or graft-vs.-host disease.

The following procedure is used to detect compounds that interact with the lipopolysaccharide in ced cytokine release from human mononuclear blood cells. The cytokines measured are interleukines 1alpha, 1beta, 6 and 8, as well as TNFalpha.

PROCEDURE

According to Böyum (1976)10 ml of freshly prepared human citrated blood is diluted 1 : 1 with PM 16-buffer (Serva, Heidelberg, FRG), and underlayered with 15 ml Lymphoprep® (Molter GmbH, Heidelberg, FRG), and subsequently centrifuged at 20 °C with 400 *g* (Minifuge®, Heraeus, Hanau, FRG) for 30 min. The cell fraction appearing as a white ring between the two phases is carefully removed by means of a syringe, diluted 1 : 1 (v/v) with PM 16 and again centrifuged for 15 min. The pellet is washed with 10 ml of RPMI 1 640 (Gibco, Berlin, FRG), containing in addition 300 mg/l L-glutamine. The washed cell fraction is taken up in 1 ml RPMI 1 640, containing in addition 300 mg/l L-glutamine, 25 mmol/l HEPES, 5% FCS and 100 IU/ml penicillin/streptomycin (Gibco). Using a cell counter (type IT, Coulter Diagnostics, Krefeld, FRG) the cell suspension which consists of about 90% lymphocytes and 10% monocytes is adjusted to approx. 5×10^9 cells/ml.

Synthesis and release of cytokines according to Tiku et al. (1986) is performed in 96 wells microtiter plates. To 0.23 ml of the cell fraction 500 ng LPS (lipopolysaccharide from Salmonella abortus equii, Sigma GmbH, Deisenhofen, FRG), dissolved in 0.01 ml dimethyl-sulfoxide/water (1 : 10, v/v), and the investigational drug, dissolved in 0.01 ml, are added. The cell suspension is now kept at 37 °C/5% CO_2 in a common incubator. Incubation time is usually 20 h (for Il-6 and Il-8 only four or one hour, respectively). The reaction is stopped by placing the microtiter plate into an ice bath. The plate is then centrifuged at 2 000 rpm for 2 min. The cytokine levels are determined in various aliquots of the supernatant using the appropriate ELISA-kit, which is commercially available from several distributors.

EVALUATION

The cytokine-concentration in the sample is determined from appropriate calibration curves. The cytokine-concentration is measured as a function of drug concentration, and related to a control experiment without drug.

In general, Hostacortin® (10 to 0,1 µmol/l) is used as the standard compound.

CRITICAL ASSESSMENT

Drugs interfering with LPS-activation, biosynthesis and cellular release of cytokines are to exhibit activity in these assay systems. Usually cell viability is not altered, as assessed by the lactate-dehydrogenase test.

MODIFICATIONS OF THE METHOD

Van der Pouw-Kraan et al. (1992) examined the regulation of interleukin (IL)-4 production by human peripheral blood T cells. Production of IL-4 as measured by ELISA was shown to be regulated differently from IL-2 and INF-γ (also measured by ELISA).

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H.3.1.8 Flow cytometric analysis of intracellular cytokines

PURPOSE AND RATIONALE

Flow cytometry is a powerful analytical technique in which individual cells can be simultaneously analyzed for several parameters, including size an granularity, as well as the expression of surface and intracellular markers defined by fluorescent antibodies. Fluorescent anti-cytokine and anti-chemokine monoclonal antibodies are very useful for the intracellular staining and multiparameter flow cytometric analysis of individual cytokine-producing cells within mixed populations. Multicolor immunofluorescent staining with antibodies against intracellular cytokines and cell surface markers provides a high resolution method to identify the nature and frequency of cells which express particular cytokines (Sander et al. 1991, 1993; Jung 1993).

PROCEDURE

Cells and cell culture

Peripheral blood is obtained from healthy human volunteers. Mononuclear cells are isolated by Ficoll gradient centrifugation. For purification of T cells or further cell sorting, mononuclear cells are incubated with neuraminidase treated sheep red blood cells and centrifuged over Ficoll. Erythrocytes are eliminated by ammonium chloride lysis. For isolation of memory cells or naive cells, T cells are incubated with a cocktail of antibodies containing anti-CD16, anti-CD56, anti-CD20, anti-CD14, (anti-CD8), and anti-CD45RA or anti-CD45R0. Cell sorting is done with the magnetic cell sorter (MACS) according to Abts et al. (1989), Miltenyi et al. (1990) using rat anti-mouse IgG1 or Ig2a antibodies labeled with superparamagnetic beads (Miltenyi, Bergisch Gladbach, Germany). Depleted cells are highly enriched with $CD4^+(CD3^+)CD45R0^+$ or $CD4^+(CD3^+)CD45R0^-CD45RA^+$ cells (>95%) and are referred to as memory cells and naive cells ($CD4^+CD45R0^-CD45RA^+$) respectively. Only depleted cells are used for experiments.

Cells (2×10^5/100 ml) are cultured in 96 well flat bottom plates for various periods of time at 37 °C and 8% CO_2 in RPMI 1 640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2×10^{-5} M mercaptoethanol and 10% AB serum. Cells are stimulated with phorbol

12-myristate 13-acetate 1–10 ng/ml + 1 μM ionomycin, phytohemagglutinin 2.4 μg/ml or phytohemagglutinin 2.4 μg/ml + phorbol 12-myristate 13-acetate 1 ng/ml in the presence or absence of 3 μM monensin (Sigma).

Staining

Cultured cells are washed twice in Hanks' balanced solution (HBSS) and then fixed in ice-cold HBSS containing 4% paraformaldehyde for 10 min. After two further washes in HBSS the cells are resuspended to 2×10^5 in 300 μl HBSS containing 0.1% saponin, 10% AB serum, 100 μg/ml goat IgG and 0.01 M HEPES buffer. After 10 min, the cells are spun down and cytokine specific antibodies diluted in HBSS with 0.1% saponin and 0.1 M HEPES buffer (saponin buffer) are added at a concentration of 1 μg/ml for 30 min at room temperature. Cells are washed twice in saponin buffer and subsequently incubated with isotype-specific second step antibodies in a concentration ranging from 0.5 to 5 μg/ml for 20 min in the dark. Cells are washed in saponin buffer and stained with streptavidin conjugates or in the case of surface staining incubated with 200 μg/ml mouse IgG diluted in saponin buffer for 15 min. After subsequent washing in saponin buffer cells are washed twice in HBSS and stained for 20 min with different antibodies in order to determine their surface phenotype. As a last step, cells are washed in HBSS.

Flow cytometry

A FACScan flow cytometer (Becton Dickinson, Mountain View, USA) equipped with a 15 mW argon ion laser and filter settings for fluorescein-isothiocyanate (530 nm), phycoerythrin (585 nm) and TRI-Color (Medac, Hamburg, Germany) or PerCP (Becton Dickinson, USA) emitting in the deep red (>650 nm) is used.

EVALUATION

5 000–10 000 cells are computed in list mode and analyzed using the FACScan research software (Becton Dickinson)

MODIFICATIONS OF THE METHOD

Slauson et al. (1999) combined the analytical power of flow cytometry with mitogen-driven, whole blood lymphocyte activation and proliferation assays to investigate the *in vitro* mechanism of action of malononitrilamides.

Protocols for immunofluorescent staining of intracellular cytokines for flow cytometric analysis are provided by BD Phar Mingen, Life Science Research Europe, Heidelberg, Germany.

Ashcroft and Lopez (2000) highlighted the opportunities in high throughput flow cytometry (HTFC) which are opened by commercial high speed machines. The specifications of these machines are cell analysis rates over 100 000 cells/s and cell sorting rates of 55 000 cells/s with high purity.

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H.3.1.9 TNF-alpha antagonism

PURPOSE AND RATIONALE

There are two distinct types of tumor necrosis factors, TNF-alpha (cachectin) and TNF-beta (lymphotoxin), with biological activities going beyond the necrosis of tumor cells. Some of the known activities include the induction of interleukin 1, activation of PMNs, modulation of endothelial cell functions, and augmentation of specific immune functions. The complex sequence of hemodynamic and metabolic collapse which leads to shock and death during lethal endotoxinemia appear to represent the response of the infected host to the acute, systemic release of TNF-alpha. Thus, drugs that antagonize the activity of this mediator could be of clinical value in combating its fatal effects.

PROCEDURE

Twenty hours before the initiation of the experiments, L 929 cells are harvested from stock cultures and are plated in 96 well culture plates (2×10^4 cells/well) and incubated at 37 °C and 5% CO_2 in air. For each group 6 wells are set up. The cells are then preincubated for 30 min with test substances or solvent before TNF-

alpha is added (between 1 and 10 IU/well). After an additional incubation time of 20 h, the culture plates are flicked out and the remaining living cells are lysed by the addition of bidistilled water (100 µl). After 30 min incubation at room temperature, 100 µl of LDH reagent are given to each culture well. After 15 min, the enzyme activity is determined photometrically at 490 nm.

EVALUATION

The percent inhibition is calculated according to the formula:

$$\% \text{ inhibition} = 100\% - \frac{\text{ext. test group} - \text{ext. spontaneous lysis}}{\text{ext. positive control} - \text{ext. spontaneous lysis}}$$

The positive control is the group which receives vehicle and TNF-alpha. The spontaneous lysis is based on cultures which receive vehicle without TNF-alpha.

MODIFICATIONS OF THE METHOD

Maloff and Delmendo (1991) measured the binding of tumor necrosis factor (TNF-α) to the human TNF receptor. Membranes were prepared from HeLa S3 human cervical epithelioid carcinoma cells. An aliquot of 0.2 mg of membrane preparation was incubated with 62 pM [^{125}I]TNF-α for 3 h at 4 °C. Nonspecific binding was measured in the presence of 50 nM TNF-α. Membranes were filtered and washed 3 times and the filters were counted to determine the bound [^{125}I]TNF-α

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H.3.1.10 Binding to interferon receptors

PURPOSE AND RATIONALE

The interferons (IFNs) were discovered in 1957 as biological agents interfering with virus replication. They are a family of secreted proteins occurring in vertebrates and can be classified as cytokines. The IFNs are multifunctional and are components of the host defense against viral and parasitic infections and certain tumors. They affect the functioning of the immune system in various ways and also affect cell proliferation and differentiation.

IFNs were initially classified by their sources as leukocyte, fibroblast, and immune IFNs. Leukocyte and fibroblast IFNs, together, were also designated as Type 1 IFNs and immune IFN as Type 2 IFN. The recent nomenclature designates leukocyte IFNs as IFN-α and IFN-ω (earlier α-1 and α-2, respectively) fibroblast IFN as IFN-β, and immune IFN as IFN-γ.

Interferons bind to receptors on the cell surface and induce the synthesis of specific proteins. Littman et al. (1985) found that recombinant IFN-γ produced in bacteria, which is not glycosylated, binds to cellular receptors with an affinity similar to that of natural IFN-γ.

PROCEDURE

Human lymphoblastoid cells (Daudi, MOLT-4 and Raji) are grown in stationary cultures in Dulbecco's medium with 10% heat-inactivated horse serum. HeLa cells are grown in Eagle's medium with 7% horse serum.

The following interferons are used: Purified recombinant interferon-γ (rIFN-γ) (Genentech, antiviral activity 1.2×10^7 units/mg); natural human INF-β (Interferon Working Group of the NCI, antiviral activity 2×10^5 roentgen units/mg); rIFN-2α (Schering, antiviral activity 2×10^8 reference units/mg); rIFN-β (Cetus Corp., antiviral activity 2.6×10^8 reference units/mg).

Fifty micrograms of rIFN-γ are reacted for 2 h at 0 °C with 1 mCi of ^{125}I-Bolton-Hunter reagent (2 000 Ci/mmol) in 0.25 ml of sodium borate buffer, pH 8.0. The reaction is stopped by the addition of glycine to a final concentration of 0.2 M and applied to a 26×0.7 cm column of Sephadex G-75 equilibrated with phosphate-buffered saline, pH 7.4, containing 0.25% gelatin. The reaction vial is washed with 20-µl aliquots of this buffer containing 40% ethylene glycol and then with buffer alone. The washes are added to the column and 0.32 ml fractions are collected. The fractions containing ^{125}I-rIFN-γ are pooled and diluted with 1/10 volume of 10-fold concentrated Eagle's medium containing 10 mg/ml bovine serum albumin and 0.1 mM dithiothreitol.

Cells harvested from exponentially growing cultures are centrifuged and resuspended at 8×10^6/ml in their own medium supplemented with 10 mM HEPES buffer, pH 7.4. Standard binding assays contain 3 to 5×10^6 cells and 0.46 nM ^{125}I-rIFN-γ. At the end of the reaction, the cells are centrifuged through 10% sucrose at 10 000 rpm in Microfuge tubes and the cell pellet is counted. A blank value is determined by incubating and processing in the same way an equal amount of ^{125}I-rIFN-γ in the absence of cells; this blank is subtracted from the cpm bound.

EVALUATION

The binding data are analyzed using the LIGAND program developed by Munson and Rodbard (1980).

MODIFICATIONS OF THE METHOD

Blatt et al. (1996) described the biological activity of consensus interferon, a wholly synthetic type I interferon, developed by scanning several interferon-alpha nonallelic subtypes and assigning the most frequently observed amino acid in each position.

IFN-τ, a new class of type I interferon was described by Pontzer et al. (1994), Alexenko et al. (1997, 1999), Martal et al. (1998), Swann et al. (1999).

Thiam et al. (1998) reported the agonist activities of a lipopeptide derived from INF-γ on murine and human cells by analysis and quantification of cell surface markers using flow cytometry and cell-ELISA.

Bosio et al. (1999) reported efficacy of type I interferon in cytomegalovirus infections *in vivo*. Oral administration of type I interferons (murine INF-α and INF-β) reduced early replication of murine cytomegalovirus in both the spleen and liver of infected BLB/c mice.

Tovey and Maury (1999) found a marked antiviral activity of murine interferon-α/β or individual recombinant species of murine INF-α, INF-β, or INF-γ or recombinant human INF-α1-8 in mice challenged systemically with a lethal dose of encephalomyocarditis virus, vesicular stomatitis virus, or varicella zoster virus. Oromucosal administration of INF-α also exerted a marked antitumor activity in mice injected i.v. with highly malignant Friend erythroleukemia cells or other transplantable tumors, such as L1 210 leukemia, the EL4 tumor, or the highly metastatic B16 melanoma.

To gain more insight into similarities of different INF-α species, Viscomi et al. (1999) evaluated neutralization and immunoactivity of a variety of INF preparations with various monoclonal antibodies obtained through immunization with recombinant, lymphoblastoid, and leukocyte INF-α.

Reporter transgenic mice expressing the luciferase gene under the control of separate TCR-response elements from the INF-γ promoter or expressing the green fluorescent protein gene under the control of an INF-γ minigene were employed by Zhang et al. (1999) to explore the basis for IL-12 regulation of INF-γ gene transcription.

Poynter and Daynes (1999) studied the influence of constitutively expressed INF-γ on age-associated alterations in inducible nitric oxide synthase regulation using cell cultures from mouse spleen for nitrite and cytokine analysis.

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H.3.1.11 Screening for interleukin-1 antagonists

PURPOSE AND RATIONALE

Interleukin-1-α and -β are potent regulators of inflammatory processes. The naturally occurring interleukin-1 receptor antagonist (IL-1ra) is effective *in vitro* and *in vivo* in modulating biological responses to IL-1 (Schreuder et al. 1995). Using a combination of anion exchange, gel filtration, and reverse-phase HPLC, three species of native IL-1ra were identified. An unglycosylated,

intracellular isoform is designed as icIL-1ra (Lennard 1995; Arend et al. 1998).

A cell-free, nonisotopic assay has been developed to discover molecules that compete with the natural ligands for binding to the active sites of the type-I interleukin-1 receptor. The key reagents are the interleukin-1 receptor antagonist, a recombinant soluble form of the receptor (sIL-1R), and a specific anti-sIL-1R nonneutralizing monoclonal antibody (Sarrubi et al. 1996).

PROCEDURE

Proteins

The extracellular portion of the type-I interleukin-1 receptor (sIL-1R) is expressed on the membrane of Chinese hamster ovary cells using a phosphatidinyl-inositol-glycan linkage (PIG-tail). Its expression, cleavage with phosphoinositol-specific phospholipase C, and purification is performed according to Whitehorn et al. (1995).

The three interleukin-1 ligands are expressed in *Escherichia coli* using synthetic genes (Dower et al. 1989; Yanofsky and Zurawski 1990). The purification of IL-1ra and IL-β is accomplished according to Schreuder et al. (1995) and Yem et al. (1988).

IL-1α is purified as follows: *E. coli* cell sonicates are precipitated with 2 M ammonium sulfate, and the pellet is resuspended in TE (25 mM Tris/HCl, pH 8.9, 1 mM EDTA), dialyzed vs. the same buffer and loaded on a DEAE-Sepharose column equilibrated with TE. The protein is eluted with a linear NaCl gradient to 300 mM. Ammonium sulfate to 0.8 M is added to the IL-1α containing fractions which are loaded onto a phenyl-Sepharose column equilibrated with TE containing 0.8 m ammonium sulfate. The elution is performed with a linear gradient to TE with no salt. IL-1α-containing fractions are concentrated and chromatographed on a Sephacryl S-200 column in PBS (phosphate buffered saline: 20 mM sodium phosphate, pH 7.3, 150 mM sodium chloride).

Fluorescein-labeled IL-1α is obtained by incubating 1 mg/ml IL-1α with 1 mg/ml fluorescein isothiocyanate in PBS for 2 h at room temperature in the dark. The reaction solution is passed directly over a G-25 column (Pharmacia) equilibrated with PBS to remove unreacted fluorescein isothiocyanate.

The monoclonal antibody Mab79 is used as direct dilutions $1:10^5$–10^6) of ascitic fluid in PBSA (PBS containing 0.3% bovine serum albumin). Horse-radish peroxidase-linked anti-mouse IgG polyclonal antibody is used.

Protein concentrations are determined using the Bio-Rad protein assay kit, based on the dye-binding procedure according to Bradford (1976). BSA is used as reference protein.

Immobilized-ligand IL-1 receptor binding assay

Essentially the same procedure can be used for both manual and automated versions of the assay, with all steps and incubations performed at room temperature. In the automated assay a Beckman Biomek 1 000 WorkStation was used for all steps, from coating to spectrophotometric measurements.

Ligand immobilization is obtained by incubation 3.6 µg/ml IL-1ra in 50 µl PBS in flat-bottomed culture-treated microplate wells, equivalent to 10 pmol/well of IL-1ra. After overnight incubation microplates are emptied and 250 µl/well of 3% BSA in PBS is added to block unreacted sites. After 2 h of incubation and three washes with an excess of PBS, the ligand-coated microplates are ready for the receptor binding reaction.

In separate microplates with U-shaped wells, 12 µl of samples (containing up to 50% DMSO or DMF) or controls (the same solution without compound) is mixed with 48 µl of 150 pM sIL-1R in PBSA. Fifty microliters of these mixtures is transferred to the IL-1ra-coated plates (equivalent to 6 fmol/well of sIL-1R) and incubated for 2 h. Microplates are then washed twice with PBS and 50 µl of 1 : 500 000 dilution of Mab79 ascitic fluid in PBSA is added to each well. After 1 h of incubation, 25 µl of 1 : 100 dilution of HRP-labeled anti-mouse IgG in PBSA is added and the incubation prolonged for an additional hour. Plates are finally washed four times with PBS and bound peroxidase activity is measured spectrophotometrically, suing either o-phenylenediamine (OPD) or tetramethylbenzidine (TMB) as substrate. In the first case, 150 µl of 1 mg/ml OPD in 0.1 M citric acid, pH 5.0, containing 0.03% of a 35% solution of hydroperoxide is added and, after color development, the reaction is stopped with 50 µl of 4.5 M sulfuric acid. Alternatively, 100 µl of 0.1 mg/ml TMB in 25 mM citric acid and 50 mM sodium phosphate, containing 0.02% hydrogen peroxide (35% solution) is added and the reaction is stopped with 50 ml of 2.5 M sulfuric acid.

EVALUATION

Absorbance (at 492 nm for OPD and 450 nm for TMB) is measured either in a Titertek microplate reader (for the manual procedure) or directly by the Biomek 1 000 WorkStation (in the automated version). IC_{50} values can be calculated from dose-response curves.

CRITICAL ASSESSMENT OF THE METHOD

Since no cells or cell membranes are used, the assay is very robust, with no interference from membrane-perturbing agents and high resistance to the organic solvent normally used to resuspend compounds of chemical libraries.

MODIFICATIONS OF THE METHOD

High affinity type I interleukin 1 receptor antagonists were discovered by screening recombinant peptide libraries (Yanosfky et al. 1996).

Akeson et al. (1996a) developed an *ex vivo* method for studying inflammation in cynomolgus monkeys using whole blood for analysis of IL-1 antagonists administered *in vivo*. Animals were given an i.v. infusion of IL-1ra, and blood samples were taken pre-infusion and during the infusion. The samples were incubated with of without IL-1β and the subsequent *ex vivo* induction of IL-6 determined. This allows the analysis of *in vivo* efficacy of antagonists without exposing the animals to IL-1.

A novel low molecular weight antagonist, selectively binding type I interleukin (IL)-1 receptor and blocking the *in vivo* responses to IL-1 was described by Akeson et al. (1996b).

Evaluation of the interleukin-1 receptor antagonist IL-1ra in a rodent abscess model of host resistance was published by Colagiovanni and Shopp (1996).

Blocking monoclonal antibodies (mAbs) specific to mouse interleukin-1 receptor antagonist (IL-1ra) were prepared by immunizing Armenian hamsters with recombinant mouse IL-1ra by Fujioka et al. (1995). A sensitive and specific ELISA against mouse IL-1ra was established.

Miesel et al. (1995) tested the anti-arthritic reactivity of the IL-1 receptor antagonist IL-1ra in male DBA/1xB10A(4R) mice with arthritis induced by intraplantar injection of potassium peroxochromate. Three µmol/kg K_3CrO_8 were administered topically into the left hind paws. One h after the induction of arthritis, 2 mg/kg IL-1ra were administered intraperitoneally which was repeated on day 2. An arthritis index was determined daily.

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H.3.1.12 Inhibition of interleukin-1β converting enzyme (ICE)

PURPOSE AND RATIONALE

Programmed cell death (apoptosis) is effected through a cascade of intracellular proteases, kwon as caspases (Alnemri et al. 1996). The interleukin-1β-converting enzyme (ICE), alternatively known as capsase-1, was the first such protein identified on the basis of sequence its homology to the pro-apoptotic *Caenorhabditis elegans* gene product, ced-3 (Yuan et al. (1993). The caspase family includes 10 reported human homologues

of ICE. By sequence homology comparisons three caspase subfamilies have been identified. The ICE subfamily includes three caspases: ICE, TX (caspase-4), and TY (caspase-5). The CPP32 subfamily includes CPP32 (caspase-3), CMH-1 (caspase-7), and MCH-2 (caspase-6). A third caspase subfamily includes ICH-1 (caspase-2), FLICE (caspase-8) and caspases-9 and -10.

ICE processes pro-IL-1β to yield active IL-1β, which plays a pivotal role in inflammatory cell activation (Dinarello 1996) and is known to inhibit the expression of apoptosis (Tatsuda et al. 1996). Inhibition of IL-1β formation is an approach for treatment of inflammatory disorders such as rheumatoid arthritis. Livingstone (1997) presented a review on *in vitro* and *in vivo* studies of peptidyl ICE inhibitors.

PROCEDURE

Neutrophil isolation

Neutrophils are isolated from healthy volunteers by dextran sedimentation and centrifugation through a discontinuous Ficoll gradient (Lee et al. 1993). Isolated neutrophils are resuspended in polypropylene tubes at a concentration of 1×10^6 cells/ml in DMEM supplemented with 10% FCS, 1% glutamine, and 1% penicillin/streptomycin solution. Neutrophil purity is assessed by size and granularity on flow cytometry.

Quantification of apoptosis

Neutrophil apoptosis is quantified by flow cytometry as the percent of cells with hypodiploid DNA (Nicoletti et al. 1991). Cells are centrifuged at 200 *g* for 10 min, gently resuspended in 500 µl of hypotonic fluorochrome solution (50 µg/ml propidium iodide, 3.4 mM sodium citrate, 1 mM Tris, 0.1 mM EDTA, and 0.1% Triton X-100) and stored in the dark at 4 °C for 3 to 4 h before analysis using a Coulter Epics XL-MCL cytofluorometer. A minimum of 5 000 events are collected and analyzed. Apoptotic nuclei are distinguished from normal neutrophil nuclei by their hypodiploid DNA; neutrophil debris is excluded from analysis by raising the forward threshold. Apoptotic nuclei appear as a broad hypodiploid DNA peak which is easily discernible from the narrow peak of cells with normal diploid DNA content. Apoptosis is assessed at 24 h after treatment.

Assay of caspase-1 activity

Cell lysates are prepared from the membrane fraction of 20×10^6 neutrophils following experimental manipulation. Aliquots of the lysates (10 µl) are diluted in assay buffer (100 mM HEPES (pH 7.4), 10% sucrose, and 0.1% 3-([3-cholamidopropyl]dimethylammonio]-1-propanesulfonate) containing 20 µM Ac-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Calbiochem) and then incubated for 45 min at room temperature. The release of 7-amino-4-methylcoumarin is detected by continuous measurement using a Perkin-Elmer LS50 luminescence spectrometer with an excitation of 380 nm and an emission slit at 460 nm. Specific ICE (caspase-1) activity is measured as pmol/s per milligram of protein.

EVALUATION

Individual experiments are repeated a minimum of four times; results are expressed as the mean ±SD. Analysis is performed using the Student *t*-test or ANOVA with Scheffè's correction.

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H.3.2 *In vivo* methods for anti-inflammatory activity

H.3.2.1 General considerations

The inflammatory process involves a series of events that can be elicited by numerous stimuli, e.g., infectious agents, ischemia, antigen-antibody interactions, chemical, thermal or mechanical injury. The response is accompanied by the clinical signs of erythema, edema, hyperalgesia and pain. Inflammatory responses

occur in three distinct phases, each apparently mediated by different mechanisms:

- an acute, transient phase, characterized by local vasodilatation and increased capillary permeability,
- a subacute phase, characterized by infiltration of leukocytes and phagocytic cells,
- and a chronic proliferative phase, in which tissue degeneration and fibrosis occur.

According to these phases, pharmacological methods have been developed.

Methods for testing acute and subacute inflammation are:

- UV-erythema in guinea pigs
- Vascular permeability
- Oxazolone-induced ear edema in mice
- Croton-oil ear edema in rats and mice
- Paw edema in rats (various modifications and various irritants)
- Pleurisy tests
- Granuloma pouch technique (various modifications and various irritants)

The proliferative phase is measured by methods for testing granuloma formation, such as:

- Cotton wool granuloma
- Glass rod granuloma
- PVC sponge granuloma.

Furthermore, methods for testing immunological factors have been developed, such as:

- Adjuvant arthritis in rats (various modifications)
- Experimental allergic encephalomyelitis
- Schultz-Dale-reaction
- Passive cutaneous anaphylaxis
- Arthus type immediate hypersensitivity
- Delayed type hypersensitivity

(see Chapter I).

H.3.2.2 Methods for testing acute and subacute inflammation

H.3.2.2.1 Ultraviolet erythema in guinea pigs

PURPOSE AND RATIONALE

The test was first described by Wilhelmi (1949) who was able to delay the development of ultraviolet erythema on albino guinea pig skin by systemic pretreatment with clinically equivalent doses of phenylbutazone and other nonsteroidal anti-inflammatory agents. The test procedure was further developed by Winder et al. (1958) and since that time modified by various investigators.

PROCEDURE

Albino guinea pigs (Pirbright white strain) of both sexes with an average weight of 350 g are used. Eighteen h prior testing, the animals are shaved on both flanks and on the back. Then they are chemically depilated by a commercial depilation product or by a suspension of barium sulfide. Twenty minutes later, the depilation paste and the fur are rinsed off in running warm water. On the next day, the test compound is dissolved (or suspended) in the vehicle and half the dose of the test compound is administered by gavage (at 10 ml/kg) 30 min before ultraviolet exposure. Control animals are treated with the vehicle alone. Four animals are used for each treatment group and control. The guinea pigs are placed in a leather cuff with a hole of 1.5×2.5 cm size punched in it, allowing the ultraviolet radiation to reach only this area. An original Hanau ultraviolet burner Q 600 is warmed up for about 30 min prior to use and placed at a constant distance (20 cm) above the animal. Following a 2 min ultraviolet exposure, the remaining half of the test compound is administered. The investigator has to protect himself/herself by gloves and ultraviolet glasses. The erythema is scored 2 and 4 h after exposure.

EVALUATION

The degree of erythema is evaluated visually by 2 different investigators in a double-blinded manner. The followings scores are given:

- 0 = no erythema,
- 1 = weak erythema,
- 2 = strong erythema,
- 4 = very strong erythema.

Animals with a score of 0 or 1 are considered to be protected. The scoring after 2 and after 4 h gives some indication of the duration of the effect. ED_{50} values can be calculated. Doses of 1.5 mg/kg indomethacin p.o., 4 mg/kg phenylbutazone p.o. and 60 mg/kg acetylsalicylic acid p.o. have been found to be effective.

CRITICAL ASSESSMENT

The test has the advantage of simplicity but needs training of the investigators. Attempts to use reflection photometers in order to eliminate subjective scoring were unsuccessful. Corticosteroids after systemic application are rather ineffective in this test, however, can be

evaluated after topical administration. The test is not particularly useful to study the duration of the anti-inflammatory effect.

MODIFICATIONS OF THE TEST

Yawalkar et al. (1991) tested several steroids after local application in the ultraviolet-induced dermatitis inhibition in guinea pigs. Clobetasol propionate was more effective than hydrocortisone, halobetasol propionate was superior to both corticosteroids.

Woodward and Owen (1979) used the albino guinea-pig ear as the site of inflammation produced by UV radiation. Ear temperature, water content of the ear and vascular permeability were measured. Indomethacin, phenylbutazone and aspirin given subcutaneously were active but paracetamol was not.

Warren et al. (1993) studied the role of nitric oxide synthase and cyclo-oxygenase in the skin blood flow to UVB irradiation in the shaved dorsal skin of anesthetized male **Sprague Dawley rats** with a laser Doppler flow probe. Topical application of clobetasol-17-propionate immediately after irradiation inhibited the 18 h UVB response in a dose-dependent manner.

Gloxhuber (1976) measured skin thickness using calipers in **hairless mice** after UV-irradiation of the back and treatment with anti-inflammatory drugs.

Woodbury et al. (1994), Kligman (1994) described a rapid assay of the anti-inflammatory activity of topical corticosteroids by inhibition of a UVA-induced neutrophil infiltration in hairless mouse skin. Skh-hairless mice were irradiated with UVA light on an area of 2×2 cm square on the dorsal trunk fore 200 min in anesthesia. Steroid treatment was once daily for 7 days. Irradiation was on the 8^{th} day. Neutrophils were counted microscopically in punch biopsies.

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H.3.2.2.2 Vascular permeability

PURPOSE AND RATIONALE

The test is used to evaluate the inhibitory activity of drugs against increased vascular permeability which is induced by a phlogistic substance (Miles and Miles 1992). Mediators of inflammation, such as histamine, prostaglandins and leucotrienes are released following stimulation e.g. of mast cells. This leads to a dilation of arterioles and venules and to an increased vascular permeability. As a consequence, fluid and plasma proteins are extravasated and edemas are formed. These effects are counteracted by H_1- antihistaminics, inhibitors of arachidonic acid metabolism and by leucotriene receptor antagonists. In addition, membrane-stabilizing drugs are able to reduce capillary permeability. Vascular permeability is increased by intracutaneous injection of the mast cell-degranulating compound 48/80. The increase of permeability can be recognized by the infiltration of the injected sites of the skin with the vital dye Evan's blue.

PROCEDURE

Male Sprague-Dawley rats with a body weight between 160 and 200 g are used. The ventral sides of the animal are shaved. Five ml/kg of an 1% solution of Evan's blue are injected intravenously. One hour later the animals are dosed with the test compound orally or intraperitoneally or with the vehicle. Ten animals are used for each test group and the control. Thirty minutes later, the animals are briefly anaesthetized with ether and 0.05 ml of an 0.01% solution of compound 48/80 are injected intracutaneously at 3 sites both at the left and ventral side. Ninety minutes after the injection of compound 48/80 the animals are sacrificed by ether anesthesia. The abdominal skin is removed and the dye-infiltrated areas of the skin are measured.

EVALUATION

The diameter of the dye-infiltrated areas is measured in millimeters in two perpendicular directions and the mean values of all injection sites in one animal are calculated. The percent inhibition in the treated animals as compared to the control group is calculated. A treated animal which shows values less than 50% of controls can be considered as positive. ED_{50} values can be calculated in this way. Phenylephrine at a dose of 15 mg/kg has been found to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The test for vascular permeability is useful for characterization of a new anti-inflammatory compound.
Since compounds with sympathomimetic activity have a pronounced effect this test cannot be regarded as a primary screening test for anti-inflammatory products. Together with an observation of writhing or "squirming" of mice, Whittle (1964) has proposed to be able to distinguish between narcotic and non narcotic analgesics.

MODIFICATIONS OF THE METHOD

Shionoya and Ohtake (1975) described a simple method for extraction of extravasated dye (Evan's blue) in the skin.

Frimmer and Müller (1962) presented a critical survey on the use of dye methods for quantitative determination of increased capillary permeability following intracutaneous injection of active substances.

McClure et al. (1992) used the Olympus CUE-2 Image Analyzer to quantify vascular permeability in the Miles assay in guinea pigs.

Zentel and Töpert (1994) used oxazolone-induced Evans blue extravasation for preclinical evaluation of topical corticosteroids. Female **NMRI-mice** were sensitized by topical application of 50 µl of 40% oxazolone in ethanol to 4 cm^2 of the left flank. After 13 days the animals were injected intravenously with 0.2 ml of 0.5% Evan's blue in water and 20 µl of 4% oxazolone in ethanol were topically applied to 6 cm^2 of the right flank immediately after injection. Three h later the challenged skin was treated with various corticosteroids in ointment. The animals were sacrificed 24 h after treatment and the challenged skin removed. Evans blue extravasation was measured spectrophotometrically at 623 nm.

Teixeira et al. (1993) studied acute inflammatory reactions in **guinea pig skin** measuring infiltration of ^{111}In-labelled eosinophils and neutrophils and edema formation by extravasation of ^{125}I-human serum albumin.

Fujii et al. (1996) quantified vascular permeability by the extravasation of pontamine sky blue in the skin of male ddY mice after subcutaneous injection of lipopolysaccharides.

Blackham and Woods (1986) measured extravasation of pontamine sky blue in the **mouse** peritoneal cavity.

Cambridge et al. (1996) investigated 6-hydroxydopamine-induced plasma extravasation in **rat skin** after intravenous injection of ^{125}I-human serum albumin and Evan's blue.

Rouleau et al. (1997) measured the inhibition of capsaicin-induced plasma extravasation by a histamine H_3 receptor agonist prodrug by analysis of extravasated Evan's blue in skin, eye conjunctiva, nasal mucosa, trachea, main bronchi, esophagus and urinary bladder of rats.

Watanabe et al. (1984) used fluorescein isothiocyanate-labeled bovine serum albumin as tracer to measure vascular permeability in the **carrageenin air pouch of rats**.

Collins et al. (1993) studied the pro-inflammatory properties of the human recombinant vascular permeability factor containing 165 amino acids in **rabbits**.

Urinary bladder cystitis induced by cyclophosphamide was used as model of intestinal inflammation and pain by several authors (Ahluwahlia et al. 1994; Bon et al. 1996; Boucher et al. 1997; Alfieri and Gardner 1997). Male Wistar rats weighing 300–400 g were treated first with test drug or saline subcutaneously or intraperitoneally and then injected 5 min later with 150 mg/kg i.p. cyclophosphamide. One h later, anaesthesia was induced by 40 mg/kg i.p. pentobarbitone and 50 mg/kg Evans blue were injected into the jugular vein. Fifteen min later, the rat was exsanguinated by infusion of 50 ml saline into the left cardiac ventricle. The urinary bladder, the left kidney, the superior lobe of the left lung and approximately 1-cm portions of the duodenum and jejunum were removed and blotted before dry weighing. The content of Evens blue dye was determined by spectrophotometry at 620 nm after extraction in known volumes of formamide at 60 °C for 60 h.

Ferrets were treated in the same way, but the dose of cyclophosphamide was 125 mg/kg and the volume of exsanguination was 300 ml.

Hirota et al. (1995) induced **chemical peritonitis in rats** by applying 0.02 M HCl on the surface of the cecum or appendix and quantified the inflammation by measuring the extravasation of intravenously injected Evan's blue bound to albumin extracted from those tissues.

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H.3.2.2.3 Inhibition of leukocyte adhesion to rat mesenteric venules *in vivo*

PURPOSE AND RATIONALE

Reversible adherence of leukocytes to endothelium, basement membranes and other surfaces is an essential event in the establishment of inflammation. Their entry into tissues is controlled by the dynamic interaction between adhesion molecules expressed by these cells and the endothelium. White cells circulating in the blood have the tendency to adhere to the walls of blood vessels and this tendency is greatly increased in states of inflammation. Normally, when leukocytes collide with the vessel wall, the collision behaves elastically and the cells bounce off and back into the lumen. However, biochemical changes in inflamed tissues results in inelastic collisions of cells and an increase in their adhesion, thus initiating rolling of leukocytes along the endothelial surface. As adhesion further increases, rolling is slowed and may be followed by the cells coming to a complete stop and their migration out of the vessel. This can be observed by preparing a mesenteric venule of an anesthetized rat and following the flow and rolling of leukocytes by means of a microscope, thus allowing in-vivo studies. In this test procedure, adhesion of leukocytes, to the vessel wall, is artificially induced by the application of the formyl-methionyl peptide fMet-Leu-Phe (FMLP). Formyl peptides are released from bacteria and mitochondria of damaged tissue, so these peptides provide a specific signal marking the presence of invading bacteria or tissue damage. The density of FMLP receptors ranges from 10^4 to 10^5 per cell, depending on the cell type. Activation of leukocytes through this receptor results in rapid expression of preformed L-selectin (LECAM-1) on the cell surface which causes the cells to roll along the endothelial surface. LECAM-1 are very rapidly shed from the surface of leukocytes, however, and integrins take over to maintain further adhesion and migration into the tissue.

PROCEDURE

Sprague-Dawley rats are anesthetized by administration of Nembutal®. The trachea, jugular vein and caro-

tid artery are prepared free, the abdominal cavity is opened and a section of ileum is pulled out and draped over a heated microscope table. Prior to test compound administration, the number of spontaneous adhering leukocytes is counted, every 5 min, in a defined section of a venule (covered with paraffin oil) during a 30-min period (control). Blood pressure, body temperature and velocity of blood flow are also registered. The test compound is administered via continuous infusion during the entire test procedure beginning at $t = -30$ min. Following the determination of control values for spontaneous adhesion, FMLP (f-Met-Leu-Phe, 10^{-4} M) is dripped twice ($t = -30$ min and $t = 0$ min) on the preparation and the number of adhering leukocytes is determined every 5 min over a 90 min period, beginning with the second application of FMLP ($t = 0$ min). Each test group consists of at least 10 animals. The mean leukocyte count of every rat prior to FMLP and/or test substance application is taken as the 100% value to obtain the baseline for further comparisons. The test compounds are dissolved in 0.9% NaCl shortly before application.

EVALUATION

Following the second topical application of FMLP (10^{-4} M), the number of adhering leukocytes in the mesenteric venule section is counted 30 min after the stimulus is given, and again at the end of the observation period of 2 h. The influence of a continuous i.v. infusion of the test drug is compared with the positive control group (FMLP stimulation, without drug).

MODIFICATIONS OF THE METHOD

A simple, rapid, *in vitro* assay for granulocyte adherence was developed by MacGregor et al. (1974). Heparinized whole blood is filtered through nylon fibers packed in Pasteur pipettes, and the percentage of granulocytes adhering was calculated.

Neutrophil adherence was tested *in vitro* by Burch et al. (1992). Human umbilical vein endothelial cells were plated at $5 \acute{} 10^4$ cells/well into collagen coated culture plates and grown to confluence. Neutrophils were labeled with ^{51}Cr. Experimental agents were added to the neutrophils before their activation with FMLP. After 15 min incubation at 37°C, the non-adherent leukocytes were removed by gentle aspiration followed by a wash with saline. The adherent neutrophils were lysed by 1 N NaOH and the radioactivity was quantitated.

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H.3.2.2.4 Oxazolone-induced ear edema in mice

PURPOSE AND RATIONALE

The oxazolone-induced ear edema model as first described by Evans (1971) in mice is a model of delayed contact hypersensitivity that permits the quantitative evaluation of the topical and systemic anti-inflammatory activity of a compound following topical administration.

PROCEDURE

Mice of either sex with a weight of 25 g are used. Before each use a fresh 2% solution of oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) in acetone is prepared. The mice are sensitized by application of 0.1 ml on the shaved abdominal skin or 0.01 ml on the inside of both ears under halothane anesthesia. The mice are challenged 8 days later again under anesthesia by applying 0.01 ml 2% oxazolone solution to the inside of the right ear (control) or 0.01 ml of oxazolone solution, in which the test compound or the standard is solved. Special pipettes of 0.1 ml or 0.01 ml are used. Groups of 10 to 15 animals are treated with the irritant alone or with the solution of the test compound. The left ear remains untreated. The maximum of inflammation occurs 24 h later. At this time the animals are sacrificed under anesthesia and a disc of 8 mm diameter is punched from both sides. The discs are immediately weighed on a balance. The weight difference is an indicator of the inflammatory edema.

EVALUATION

Average values of the increase of weight are calculated for each treated group and compared statistically with the control group. A 0.003% solution of hydrocortisone and a 1% solution of indomethacin were found to be active.

CRITICAL ASSESSMENT OF THE METHOD

The method is suitable for both steroidal and non-steroidal compounds as well as for the evaluation of various topical formulations

MODIFICATIONS OF THE METHOD

Griswold et al. (1974) applied a 3% solution of oxazolone to the left paw of mice. The edema was assessed plethysmographically

Various cutaneous models of inflammation for the evaluation of topical and systemic pharmacological agents have been discussed by Young and Young (1989).

Bailey et al. (1995) described a contact hypersensitivity model in mice for rank-ordering formulated corticosteroids. Male Swiss Webster mice were sensitized with 20 µl of 2% oxazolone on the inner and outer aspects of each ear (10 µl each side). Mice were challenged 7 days later with 2% oxazolone in acetone : olive oil (4 : 1) on both sides of the right ear. Animals were topically treated with corticosteroids or non-steroidal antiinflammatory drugs or 20 mg formulated corticosteroids immediately after challenge. The mice were sacrificed after 24 h and edema and myeloperoxidase activity were determined. Edema was measured by taking the weight of 6 mm trephine punch biopsies of the right and left ears. Inhibition was calculated from change in ear weight of control or drug treated ears versus placebo treated ears. Myeloperoxidase activity was assessed spectrophotometrically (Williams et al. 1983) on tissue homogenates. In a delayed-type hypersensitivity model animals were treated as in the contact hypersensitivity model, except the mice were sensitized with 40 µl of 2% oxazolone in acetone:olive oil (4 : 1) on the unshaved inguinal areas.

Meingassner et al. (1997) studied anti-inflammatory activity using allergic contact dermatitis im **mice, rats** and **pigs**. Mice were sensitized on the shaved abdomen with 50 µl of 2% oxazolone solution in acetone. After 7 days, they were challenged with 10 µl of 2% (for topical testing) or 0.5% (for systemic testing) oxazolone on the inner surface of the right ears. Pinnal weight was taken as a measure of inflammatory edema 24 h after challenge. Female Sprague Dawley *rats* were sensitized by application of 80 µl of 2,4-dinitrofluorobenzene solution applied in 20 µl volumes to the inner surface of both ear lobes and to both shaved inguinal regions on day 1. Allergic contact dermatitis was elicited with 30 µl of 0.5% 2,4-dinitrofluorobenzene applied to the test sites of ≈15 mm in diameter on both shaved flanks on day 12. Animals were treated by gavage 2 h before and immediately after challenge. Dermatitis was evaluated by measuring the thickness of the lifted skin fold at the test sites with a spring-loaded micrometer. Domestic **pigs** were sensitized wit 400 µl of 10% 2,4-dinitrofluorobenzene applied to four areas on both ears and groins. Challenge reactions were elicited 12 days later with 15 µl of 2,4-dinitrofluorobenzene (1%) applied topically to test sites arranged in four craniocaudal lines on the dorsolateral shaved back (24 or 32 per pig). Test sites were treated twice either with 20 µl solution of test compound or with ≈50 mg of a cream formulation applied topically 30 min and 6 h after challenge. One day after challenge the test sites were visually evaluated for intensity and extent of erythema and induration.

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H.3.2.2.5 Croton-oil ear edema in rats and mice

PURPOSE AND RATIONALE

The method has been developed primarily as a bioassay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied steroids by Tonelli et al. (1965)

PROCEDURE

For tests in mice the irritant is composed as follows (v/v): 1 part Croton oil, 10 parts ethanol, 20 parts pyridine, 69 parts ethyl ether. For tests in rats the following mixture is prepared (v/v): 4 parts Croton oil, 10 parts ethanol, 20 parts pyridine, 66 parts ethyl ether. The standards and the test compounds are dissolved in

this solution. For tests in mice male NMRI-mice with an weight of 22 g, for tests in rats male Sprague-Dawley rats with a weight of 70 g are used. Ten animals are used for controls and each test group. The test compounds are dissolved in a concentration of 0.03 mg/ml to 1 mg/ml for mice and in a 3 to 10 times higher concentration for rats in the irritant solution. On both sides of the right ear 0.01 ml in mice or 0.02 ml in rats are applied. Controls receive only the irritant solvent. The left ear remains untreated. The irritant is applied under ether anesthesia. Four hours after application the animals are sacrificed under anesthesia. Both ears are removed and discs of 8 mm diameter are punched. The discs are weighed immediately and the weight difference between the treated and untreated ear is recorded indicating the degree of inflammatory edema. In the originally described method the ears are removed by sharp, straight scissors 6 h after application and weighed as total. The animals were sacrificed 48 h after topical ad-ministration and the thymus glands were removed, weighed and expressed as mg thymus/100 g body weight.

EVALUATION

The antiphlogistic effect can be determined by expressing the increase in weight of the treated ear as percentage of the weight of the contralateral control ear. The difference of both weights is divided by the weight of the contralateral ear times 100. Otherwise, the difference between both ears or excised discs is calculated as the average values for treated and control groups and the effect is evaluated by statistical methods. Concentration of 0.5 to 1 mg/ml hydrocortisone have been proven to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The method is useful for evaluation of anti-inflammatory topical steroids especially in the modification when thymus weight is determined simultaneously. The method also can be used for topically applied nonsteroidal antiphlogistics.

MODIFICATIONS OF THE METHOD

Wilhelmi and Domenjoz (1951) tested various drugs using Croton oil induced ear edema in mice and rabbits.

Tubaro et al. (1985) tested various anti-inflammatory drugs in the Croton oil test in mice. Granulocyte infiltration in plugs taken from the inflamed ears was assessed by measuring peroxidase activity.

Zentel and Töpert (1994) used Croton oil-induced ear edema in rats to evaluate topical corticosteroids. A plastic collar was fixed around the neck of Wistar rats of either sex (160–200 g body weight) to exclude oral uptake of the compounds. Fifty µl of 5% Croton oil in ethanol or ethanol alone were topically applied to both ears. In the treatment groups drugs were coapplied with Croton oil. Five h after treatment the animals were sacrificed by CO_2 gas and the ears removed. Edema formation was measured by the increase in wet weight.

Iwasaki et al. (1995) measured the inhibition of Croton oil-induced ear edema in Wistar rats by locally applied clobetasol-17-propionate, a synthetic glucocorticoid, and the influence of simultaneously applied RU 486.

Weirich et al. (1977) measured skin temperature, ear thickness and weight of excised punches after Croton oil induced edema in the ears of **white rabbits** and calculated phlogostasis values as the products of the percent reduction in skin temperature, auricular thickness and tissue weight in relation to controls. The authors recommended this model for the primary evaluation of topical anti-inflammatory agents.

Colorado et al. (1991) described an apparatus to measure Croton oil induced ear edema in mice using precisely reproducible pressure on the ear. The device allows to follow the time course of inflammation by repeated measurements.

Akiyama et al. (1994) studied staphylococcus aureus infection on experimental Croton oil-inflamed skin in mice. Staphylococcus aureus cells were inoculated on the surface of skin inflamed by application of Croton oil in cyclophosphamide-treated mice. Skin specimens were taken at 1, 3, 6, 12, and 24 h after inoculation and examined by microscopy. The staphylococcus aureus cells which attached to the surface of the skin immediately after inoculation had invaded the horny layer within 1 h. The cells gradually penetrated deeper into the epidermis. Application of corticosteroid ointments decreased the number of staphylococcus aureus cells in the lesions.

Anderson and Groth (1984) induced **toxic contact reactions** to Croton oil or dinitrochlorobenzene (DNCB), or **allergic contact reactions** to DNCB or oxazolone in guinea pig skin and tested the effect of various locally applied corticosteroids by macroscopic assessment and microscopic evaluation of cellular infiltrates.

Tarayre et al. (1984) used a 0.25% solution of **cantharidin** in acetone and applied 0.025 ml to one mouse ear. Two phases of inflammation were observed. After local application nonsteroidal drugs showed effects in the first phase only, whereas steroids influenced both phases.

De Young et al. (1987) induced ear inflammation in rats by intradermal injection of 10 ng **recombinant human interleukin-1β** in 10 µl of saline.

Maloff et al. (1989) injected 20 µl of interleukin-1 solution into the left ear of mice and found a dose-dependent increase of ear thickness and myeloperoxidase activity which reached the maximum after 24 h. These

effects were reduced by high doses of glucocorticoids but not by nonsteroidal anti-inflammatory drugs.

Chang et al. (1987) applied 4 µg **tetradecanoyl phorbol acetate** and test drugs dissolved in acetone to the right ear of mice. Ear edema was calculated by subtracting the thickness of the left ear (vehicle control) from the right ear (treated ear).

De Young et al. (1989) examined the temporal patterns of edema and accumulation of the polymorphic nuclear cell marker enzyme myeloperoxidase following application of tetradecanoyl phorbol acetate to mouse ears. Topical and oral corticosteroids inhibited both edema and myeloperoxidase accumulation, whereby clobetasol propionate was more effective than fluocinolone and dexamethasone. Cyclo-oxygenase and lipoxygenase inhibitors were very effective against myeloperoxidase accumulation but were inactive or moderately active vs. edema.

Topical application of arachidonic acid to mouse ear has become a widely used test (Young et al. 1983, 1984; Opas et al. 1985; Crummey et al. 1987; Hensby et al. 1987; Tomchek et al. 1991) One mg arachidonic acid is applied to the right ear of mice and vehicle to the left ear of each animal. Drugs are topically applied in acetone to the ear 30 min prior to the arachidonic acid application. Ear swelling was measured using a caliper one hour after arachidonic acid.

Griswold et al. (1995) induced inflammation in mice by local application of arachidonic acid or phorbol ester. Besides ear thickness, myeloperoxidase and DNA content was measured.

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H.3.2.2.6 Paw edema

PURPOSE AND RATIONALE

Among the many methods used for screening of antiinflammatory drugs, one of the most commonly em-

ployed techniques is based upon the ability of such agents to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, Aerosil®, sulfated polysaccharides like carrageenin or naphthoylheparamine. The effect can be measured in several ways. The hind limb can be dissected at the talocrural joint and weighed. Usually, the volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to the controls. Many methods have been described how to measure the paw volume by simple and less accurate and by more sophisticated electronically devised methods. The value of the assessment is less dependent on the apparatus but much more on the irritant being chosen. Some irritants induce only a short lasting inflammation whereas other irritants cause the paw edema to continue over more than 24 h.

PROCEDURE

Male or female Sprague-Dawley rats with a body weight between 100 and 150 g are used. The animals are starved overnight. To insure uniform hydration, the rats receive 5 ml of water by stomach tube (controls) or the test drug dissolved or suspended in the same volume. Thirty minutes later, the rats are challenged by a subcutaneous injection of 0.05 ml of 1% solution of carrageenan into the plantar side of the left hind paw. The paw is marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark. The paw volume is measured plethysmographically immediately after injection, again 3 and 6 h, and eventually 24 h after challenge.

Apparatus

Various devices have been developed for plethysmography of the paw. Winter et al. (1963) used mercury for immersion of the paw. A more sophisticated apparatus has been described by Hofrichter et al. (1969). Alpermann and Magerkurth (1972) described an apparatus based on the principle of transforming the volume being increased by immersion of the paw into a proportional voltage using a pressure transducer. Webb and Griswold (1984) reported a sensitive method of measuring mouse paw volume by interfacing a Mettler DeltaRange top-loading balance with a microcomputer. Several authors used a commercially available plethysmometer from Ugo Basile, Varese, Italy (Damas and Remacle-Volon 1992; Braga da Motta et al. 1994; Legat et al. 1994; Griesbacher et al. 1994).

EVALUATION

The increase of paw volume after 3 or 6 h is calculated as percentage compared with the volume measured immediately after injection of the irritant for each animal. Effectively treated animals show much less edema. The difference of average values between treated animals and control groups is calculated for each time interval and statistically evaluated. The difference at the various time intervals give some hints for the duration of the anti-inflammatory effect. A dose- response curve is run for active drugs and ED_{50} values can be determined.

MODIFICATIONS

Many agents can be used as irritants to induce paw edema in rats or mice. These are:

- 0.05 ml undiluted fresh egg white (Randall and Baruth 1976)
- 0.1 ml of 1% ovalbumin solution (Turner 1965)
- 0.1 ml of 1% formalin (Turner 1965)
- 0.1 ml of 0.2% carrageenan solution (Schönhöfer 1967)
- 0.1 ml of 1% carrageenan solution plus 100 ng PGE_2 or PGI_2 (Higgs et al. 1978; Portanova et al. 1996)
- 0.1 ml of 1 to 3% dextran solution (Turner 1965)
- 0.1 ml of 2.5% brewer's yeast powder suspension (Tsumuri et al. 1986)
- 0.1 ml of 0.5% ß-naphthoylheparamine solution (Peterfalvi et al. 1966)
- 0.1 ml of 0.1% trypsin solution (Kalbhen and Smalla 1977)
- 0.1 ml of 0.1% collagenase solution (Souza Pinto et al. 1995)
- 0.1 ml of 0.1% solution of collagenase from *Clostridium histolyticum* (Legat et al. 1994)
- 0.1 ml of solution of 100 IU hyaluronidase (Dewes 1955, Kalbhen and Smalla 1977)
- 0.1 ml of complete Freund's adjuvant
- 0.05 ml of 0.02% serotonin solution (Kalbhen and Smalla 1977)
- 0.1 ml of 0.005% bradykinin solution (Damas and Remacle-Volon 1992)
- 0.1 ml of 0.1 mg/ml prostaglandin E2 (Nikolov et al. 1978)
- 0.1 ml of 2.0 µg/ml prostaglandin E2 (repeated injections, Willis and Cornelsen 1973)
- 0.1 ml of 1% concanavalin A solution (Lewis et al. 1976)
- 0.1 ml of 2.5% suspension of Aerosil®
- 0.1 ml of 5% suspension of kaolin (Lorenz 1961; Wagner-Jauregg et al. 1962)
- 0.05 ml of bentonite gel (Marek 1980)
- 0.1 ml of nystatin 15 000 units (Schiatti et al. 1970, Arrigoni-Martelli et al. 1971)
- 0.1 ml of 1% phytohaemagglutinin-P solution (Lewis et al. 1976)

0.01 ml of 0.5% adriamycin (mouse paw) (Siegel et al. 1980)
0.1 ml of 0.001–0.1% solutions of various phospholipases A2 (Cirino et al. 1989)
0.1 ml of 0.1% Zymosan solution (Gemmell et al. 1979)
0.1 ml of 0.05% anti-IgG solution (Gemmell et al. 1979)
0.1 ml of 2.5% mustard powder suspension (Tsumuri et al. 1986)
0.1 ml of solution containing 1 unit of cobra venom factor (Leyck and Parnham 1990)
0.05 ml of 0.02–0.2% sonic extract from *Porphyromonas gingivalis* (Griesbacher et al. 1994)
0.1 ml of 0.25% suspension of papaya latex (Gupta et al. 1994)

The edema induced by the various irritants lasts for different times such as a few hours after serotonin and up to 2 days after Aerosil® or after kaolin. These irritants therefore are suitable to study not only the degree but also the duration of the anti-inflammatory action.

Standards

Depending on the irritant steroidal and nonsteroidal anti-inflammatory drugs have a pronounced effect in the paw edema test. With carrageenan as irritant doses of 50 to 100 mg/kg phenylbutazone p.o. have been found to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The paw edema method has been used by many investigators and has been proven to be suitable for screening purposes as well as for more in depth evaluations. Dependent on the irritant steroidal and nonsteroidal anti-inflammatory drugs, antihistaminics and also, to a lesser degree, serotonin antagonists are active in the paw edema tests. Since so many different irritants have been used by the various investigators the results are often difficult to compare.

FURTHER MODIFICATIONS OF THE METHOD

Besides paw volume Shirota et al. (1984) determined the surface temperature of the inflamed paw in rats using a special cage with rolling rods.

Brooks et al. (1991) used anesthetized **dogs** and demonstrated that a significant inflammatory response can be elicited in the dog paw by subcutaneous injection of carrageenan. The increase in paw volume can be quantitatively measured as a pressure change recorded via a water-filled balloon fixed against the paw with nonexpandable tape. Effective doses of non-steroidal antiinflammatory drugs were closer to human therapeutic doses in dogs than in rats.

Oyanagui and Sato (1991) described an **ischemic paw assay in mice**. A commercial rubber ring (1×1 mm, $d = 42$ mm) was bound 14 times to the right hind leg of mice just above the articulation. After 20 min of ischemia, the rubber was cut off with scissors. Paw swelling was measured after an other 20 min of natural blood recirculation.

Wirth et al. (1992) described a **thermic edema** which was induced in anesthetized Sprague-Dawley rats by immersing paws of the right and left hindlimb into water of 55 °C. Immediately thereafter, the rats received the test drug (the bradykinin antagonist Hoe 140) intravenously. Paw volume was measured at regular intervals by plethysmography.

Braga da Motta et al. (1994) described drug modulation of **antigen-induced paw edema in guinea-pigs**. Male short-haired guinea pigs weighing 250–350 g received on day 0 a single dorsal s.c. injection of 1 ml of phosphate buffered saline containing 20 µg of ovalbumin, dispersed in 1 mg $Al(OH)_3$. The animals were boosted with a similar injection of antigen on days 14, 21, and 28. Thirty five days after the first injection of antigen or $Al(OH)_3$, the animals received an intraplantar injection of 0.5, 5, 50, or 200 µg ovalbumin, diluted in 100 µl of phosphate buffered saline. Edema was measured 2, 4, 6, 8, 24, and 48 h after the challenge.

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H.3.2.2.7 Pleurisy test

PURPOSE AND RATIONALE

Pleurisy is a well known phenomenon of exudative inflammation in man. In experimental animals pleurisy can be induced by several irritants, such as histamine, bradykinin, prostaglandins, mast cell degranulators, dextran, enzymes, antigens, microbes, and nonspecific irritants, like turpentine and carrageenan (Survey by DeBrito 1989). Carrageenan-induced pleurisy in rats is considered to be an excellent acute inflammatory model in which fluid extravasation, leukocyte migration and the various biochemical parameters involved in the inflammatory response can be measured easily in the exudate.

PROCEDURE

Male Sprague-Dawley rats weighing 220–260 g are used. The animal is lightly anaesthetized with ether, placed on its back and the hair from skin over the ribs of the right side is removed using animal clippers. The region is swabbed with alcohol. A small incision is made into the skin under the right arm between the seventh and eighth rib. The wound is opened and a further shallow incision is made into the exposed intercostal muscle. 0.1 ml of 2% carrageenin solution is injected into the pleural cavity through this incision. The injection needs to be made swiftly to avoid the risk of injuring the lung. The wound is closed with a Michel clip.

One hour before carrageenan injection and 24 and 48 h thereafter, groups of 10 rats are treated with the standard or the test compound subcutaneously or orally. A control group receives only the vehicle of medication. The animals are sacrificed 72 h after carrageenin injection by ether inhalation. The animal is pinned on a dissection board with the forelimbs fully extended. An incision in the skin over the xiphosternal cartilage is made to free the cartilage from overlying connective tissue. The cartilage is lifted with a forceps and a small cut is made with scissors in the body wall below to gain access into the pleural cavity. One ml of heparinized Hank's solution is injected into the pleural cavity through this cut. The cavity is gently massaged to mix its contents. The fluid is aspirated out of the cavity using a pipette. This is made easier if the dissection board is raised to an angle of 45–60°; the contents then pool in the corners of the cavity. The aspirated exudate is collected in a graduated plastic tube.

EVALUATION

One ml (the added Hank's solution) is subtracted from the measured volume. The values of each experimental group are averaged and compared with the control group. ED_{50} values can be calculated using various doses. Several other parameters can be used:

- Measuring the white blood cell number in the exudate using a Coulter counter or a hematocytometer,
- Determination of lysosomal enzyme activities,
- Determination of fibronectin,
- Determination of PgE_2.

CRITICAL ASSESSMENT OF THE METHOD

The pleurisy model has been accepted as a reliable method to study acute and subacute inflammation allowing the determination of several parameters simultaneously or successively. The activity of steroids as well as of non-steroidal drugs can be measured (Tomlinson et al. 1994; Harada et al. 1996).

MODIFICATIONS OF THE METHOD

The Evans blue-carrageenan-induced pleural effusion model has been proposed by Sancilio (1969, 1973) for screening of compounds with anti-inflammatory activity.

Meyers et al. (1993) tested the effect of treatment with interleukin-1 receptor antagonist on the development of carrageenan-induced pleurisy in intact and adrenalectomized rats.

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H.3.2.2.8 Granuloma pouch technique

PURPOSE AND RATIONALE

The method originally invented by Selye has been developed for screening by Robert and Nezamis (1957) using croton oil as irritant. An aseptic inflammation resulting in large volumes of hemorrhage exudate is elicited which resembles the subacute type of inflammation. Instead of croton oil carrageenan can be used as irritant.

PROCEDURE

Male or female Sprague-Dawley rats with a body weight between 150 and 200 g are used. Ten animals are taken for controls and for test groups. The back of the animals is shaved and disinfected. With a very thin needle a pneumoderma is made in the middle of the dorsal skin by injection of 20 ml of air under ether anesthesia. Into the resulting oval airpouch 0.5 ml of a 1% solution of Croton oil in sesame oil is injected avoiding any leakage of air. Forty-eight hours later the air is withdrawn from the pouch and 72 h later any resulting adhesions are broken. Instead of croton oil 1 ml of a 20% suspension of carrageenan in sesame oil can be used as irritant. Starting with the formation of the pouch, the animals are treated every day either orally or subcutaneously with the test compound or the standard. For testing local activity, the test compound is injected directly into the air sac at the same time as the irritant. On the 4^{th} or the 5^{th} day the animals are sacrificed under anesthesia. The pouch is opened and the exudate is collected in glass cylinders. Controls have an exudate volume between 6 and 12 ml, which is reduced dose dependent in the treated animals.

EVALUATION

The average value of the exudate of the controls and the test groups is calculated. Comparison is made by statistical means. A clear dose response curve could be found by s.c. injection of 0.5, 1.0 and 2.0 mg hydrocortisone acetate/rat. Also doses of 1.5 mg/kg indomethacin were found to be active.

CRITICAL ASSESSMENT OF THE METHOD

The method has been very useful to estimate the potency of anti-inflammatory corticosteroids both after local and after systemic application. By injection of a depot-preparation and induction of the granuloma pouch after various time intervals up to 4 weeks the duration of action can also be determined (Vogel 1963, 1965).

MODIFICATIONS OF THE METHOD

Carrageenin was used to induce exudate formation (Boris and Stevenson 1965).

Bobalik and Bastian (1967) developed a modified granuloma pouch technique in which *Mycobacterium butyricum* (adjuvant) was used as phlogistic agent.

Moreno (1993) sensitized rats by subcutaneous injection of methylated bovine serum albumin emulsified in complete Freund's adjuvant one week prior to the preparation of air pouches which were reinflated 4 days later. Seven days after formation of the air pouches inflammation was induced in the pouches by injection of 1 mg methylated bovine serum albumin.

Martin et al. (1994) described an air pouch model in the 6-day-old rat by injection of carrageenan. Besides the usual parameters, leukocyte influx and the level of prostaglandin E_2 in the pouch exudate were measured.

In order to measure the effects of different classes of proteinase inhibitors, Karran and Harper (1995) studied collagen degradation in subcutaneous air pouches in rats. The air pouches were formed in the dorsal region and were inflamed 6 to 8 days later by injecting λ-type carrageenan. Degradation of ^{14}C-collagen was followed in the inflammatory exudate fluid of the air pouches.

Sugio and Tsurufuji S (1981) re-evaluated the vascular constriction hypothesis as the mechanism of anti-inflammatory action of glucocorticoids. Rats were injected with 8 ml of air subcutaneously on the dorsal sur-face under light ether anesthesia to make an oval air sac. After 24 h, 4.0 ml of 2% heat-sterilized solution of carrageenin in 0.9% NaCl solution was injected into the air sac (day 0). Drug effects were tested on day 7. Vascular permeability in the granuloma pouch was measured using ^{125}I-HSA and ^{131}I-HSA. About 1 μCi of purified ^{125}I-HSA in 0.2 ml saline was injected into the femoral vein. After 30 min, 1.0 ml of the pouch fluid was withdrawn to measure the leakage of ^{125}I-HSA into the pouch fluid. After administration of the drug, about 1 μCi of purified ^{131}I-HSA was injected into the femoral vein. After 30 min, 1.0 ml of the pouch fluid was again withdrawn to measure the concentration of ^{131}I-HSA. The ratio of ^{125}I-HSA/^{131}I-HSA was taken as an index of vascular permeability change induced by drug treatment.

Atkinson et al. (1962) implanted compressed pellets of carrageenin subcutaneously to rats and measured the effects of some anti-inflammatory substances on wet weight of the pellets.

Bowers et al. (1985) described a method to induced a **granuloma in the rat lung** by instillation of a 2% carrageenan solution into one lower lobe of the lung via the trachea. No respiratory impairment was noticed during this procedure.

Further phlogistic agents inducing specific inflammatory cascades such as zymosan (complement activation) or lipopolysaccharide (cytokine release) have been used for pharmacological evaluation of antiinflammatory agents (Erdö 1994; Miller 1997).

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H.3.2.2.9 Urate-induced synovitis

PURPOSE AND RATIONALE

The importance of urate in gout and the deposition of sodium urate in gouty tophi is well known. Faires and McCarty (1962) reported that they themselves were the subjects for a study injecting 20 mg sodium urate crystal suspension in their own knee-joint. They experienced severe pain and prostration which resembled an acute gouty attack. Based on this experience they developed an experimental model in dogs for testing anti-inflammatory compounds (McCarty et al. 1963, 1966).

PROCEDURE

Preparation of sodium urate crystals

0.4 g (0.01 Mol) sodium hydroxide pellets are dissolved in 400 ml distilled water in a glass beaker; 1.68 g (0.01 Mol) uric acid is added. The resultant opaque preparation is allowed to remain overnight at room temperature. The next morning, the crystals are harvested by decanting the supernatant solution and are then washed 3 times in cold saline, resuspended in saline and sterilized in an autoclave. Suspensions for injections are kept in rubber-stoppered, multi-dose vials containing 15 to 24 mg of urate per ml.

Unanesthetized healthy dogs weighing between 18 and 25 kg are used. They are trained to lie quietly on their backs in a dog cradle under light restraint. The skin above one knee is shaved, disinfected and a sterile 21-gauge needle inserted into the joint. Slight aspiration produces a small amount of clear, viscous synovial fluid, indicating entry into the joint. The needle is left in place, a syringe containing the urate suspension is attached and volumes from 0.1 to 0.5 ml are injected into the joint (approximately 2–10 mg urate).

One hour before the injection of urate crystals the animals are treated with the test compound or the standard. Experiments are designed so that a pair of dogs is tested on each of 2 days. On the first day, only one dog receives the drug. One week later the opposite knee of each dog is injected, but the other dog is treated.

EVALUATION

A scoring system is adopted in which inflammatory symptoms ranging from tenderness, limping, occasional 3-legged gait to complete 3-legged gait are scored from 1+ to 4+.

CRITICAL ASSESSMENT OF THE METHOD

In spite of the fact that the experiment originally has been performed in human volunteers and that the method closely resembles pathological conditions in man, due to animal protection law conditions the method can be recommended only for special investigations.

MODIFICATIONS OF THE METHOD

Carlson et al. (1986) developed an automated microcomputer-based system for determining canine paw pressure quantitatively in the dog synovitis model.

According to Phelps et al. (1967) dogs are anesthetized and placed on their sides with the hind leg firmly fixed with tape so that the femur and tibia form a 90-degree angle. The knee is punctured with a needle. When a few drops of synovial fluid can be withdrawn indicating a correct puncture of the joint, 6–10 ml of saline are injected to distend the joint and a polyethylene catheter is inserted through the needle, which is then withdrawn. 0.5 ml of a 0.02% sodium urate suspension are injected into the joint. The catheter is attached to a pressure transducer. Constant pressure recordings can be taken during the acute phase of inflammation. Pressure changes are plotted against time, whereby each dog is compared with his own control. Treatment with nonsteroidal anti-inflammatory drugs, such as indomethacin, show a considerable reduction of intraarticular pressure.

Fujihira et al. (1971) injected the urate suspension into the knee joint of a hind leg of well trained Beagle dogs. They were placed on three weighing machines whereby both forelegs rested on one balance, and the hindlegs individually on other balances. In this way, the relative change of weight on each hindleg after intraarticular injection of urate suspension can be measured, indicating a decrease of weight in the injected leg, counterbalanced to the other leg. Time response curves could be found after non-steroidal anti-inflammatory drugs.

Rosenthale et al. (1972) found a long-lasting inflammatory effect of prostaglandins PGE_1 and PGE_2 after injection in the knee joint of dogs.

Schaible and Schmidt (1985) induced an acute experimental arthritis in the knee joint of anesthetized **cats** by intraarticular injection of a 4% kaolin suspension and recorded the activity of single fine afferent units from filaments of the saphenous nerve.

Perkins and Campell (1992) injected either sodium urate crystals or Freund's complete adjuvant into one knee of **rats**. The maximum tolerated pressure was determined with or without treatment by analgesic drugs after 18–24 h (urate injections) or 64–70 h (Freund's complete adjuvant).

Daniel and Jouvin (1984) induced inflammation of the **guinea pig palatal mucosa** by injection of a microcrystalline suspension of monosodium urate.

Botrel et al. (1994) induced chronic inflammation in the knee joint of Beagle dogs by intra-articular injection of Freund's complete adjuvant. Besides body temperature, differences in skin temperature, difference in stifle diameter, the vertical force exerted by the arthritic hind limb measured by a force plate was chosen as parameter.

Schött et al. (1994) induced monoarthritis in rats by injection of 300 µg carrageenan in 50 µl saline into the right tibio-tarsal joint. Weight bearing was found to be an objective measure of arthritic pain.

Carleson et al. (1996) induced acute inflammation in the temporomandibular joint of rats by intraarticular injection of substance P and measured neurokinin A, calcitonin gene-related peptide and neuropeptide Y in the perfusate.

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H.3.2.3 Methods for testing the proliferative phase (granuloma formation)

H.3.2.3.1 Cotton wool granuloma

PURPOSE AND RATIONALE

The method has been described first by Meier et al. (1950) who showed that foreign body granulomas were provoked in rats by subcutaneous implantation of pellets of compressed cotton. After several days, histologically giant cells and undifferentiated connective tissue can be observed besides the fluid infiltration. The amount of newly formed connective tissue can be measured by weighing the dried pellets after removal. More intensive granuloma formation has been observed if the cotton pellets have been impregnated with carrageenin.

PROCEDURE

Male Wistar rats with an average weight of 200 g are anaesthetized with ether. The back skin is shaved and disinfected with 70% ethanol. An incision is made in the lumbar region. By a blunted forceps subcutaneous tunnels are formed and a sterilized cotton pellet is placed on both sides in the scapular region. The pellets are either standardized for use in dentistry weighing 20 mg or pellets formed from raw cotton which produce a more pronounced inflammation than bleached cotton. The animals are treated for 7 days subcutaneously or orally. Than, the animals are sacrificed, the pellets prepared and dried until the weight remains constant. The net dry weight, i.e. after subtracting the weight of the cotton pellet is determined.

EVALUATION

The average weight of the pellets of the control group as well as of the test group is calculated. The percent change of granuloma weight relative to vehicle control group is determined.

CRITICAL ASSESSMENT OF THE METHOD

The method has been useful for evaluation of steroidal and nonsteroidal anti-inflammatory drugs. For testing corticosteroids, the test can be performed in adrenalectomized rats.

MODIFICATIONS OF THE METHOD

Bush and Alexander (1960) produced granulomata in rats by means of cotton-wool pellets which have been impregnated with carrageenin.

Tanaka et al. (1960) implanted filter paper pellets soaked with 7% formalin solution in rats.

Hicks (1969) implanted pellets impregnated with irritant substances, such as capsicum oleoresin.

Instead of cotton pellets, paper disks have been implanted (Tsurumi et al. 1986).

Roszkowski et al. (1971) immersed the cotton pellets in a 1% carrageenan solution, dried overnight and soaked in a 0.25 oxytetracycline solution before implantation.

D'Arcy and Howard (1967) induced a localized inflammatory reaction in the chorio-allantoic membrane of the chick embryo by the implantation of a sterile filter paper disc, followed by re-incubation *in situ* for 4 days.

Rudas (1960) described a method for quantitative evaluation of the granulation tissue formed in experimental wounds. Plastic rings were incorporated into the wounds on the back of rats inhibiting contraction of the wound edges and epithelialization of the wound. The growth of granulation tissue inside the rings was measured.

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H.3.2.3.2 Sponge implantation technique

PURPOSE AND RATIONALE

The sponge implantation technique was described first by Saxena (1960) for short term experiments but was used subsequently to study the formation of granulomata using long-term implantation.

PROCEDURE

Sponges used for implantation are prepared from polyvinyl foam sheets (thickness 5 mm). Discs are punched out to a standard size and weight (10.0 ±0.02 mg) using a 13 mm cork borer. The sponges are then soaked in 70% v/v ethanol for 30 min, rinsed four times with distilled water and heated at 80 °C for 2 h. Prior to implantation in the animal, the sponges are soaked in sterile 0.9% saline in which either drugs, antigens or irritants have been suspended. Typical examples include 1% carrageenan, 1% yeast, 1% Zymosan A, 6% dextran, heat killed *Bordetella pertussis* (4×10^9 to 5×10^{10} organisms/ml) or 0.5% heat killed mycobacterium tuberculosis.

Sponges are implanted in female Wistar rats weighing 150–200 g under ether anesthesia. A 20 mm dorsal incision is made and the dermis separated from the underlying muscle layer by insertion of blunt forceps to form separate cavities into which sponges are inserted. Up to 8 sponges may be implanted per rat. The dorsal incision is closed with Michel clips and the animals are maintained at a constant temperature of 24 °C.

For short term experiments, the animals are treated with test drug or standard once before implantation orally or subcutaneously. For long term experiments, the rats are treated daily up to 3 weeks.

EVALUATION

For estimation of the fluid phase of sponge exudates, e.g. protein content, enzyme levels and biological mediators such as prostaglandins as well as for leukocyte migration, the sponges are removed already after 9 h.

For studying the chronic phase of inflammation besides dry weight DNA, indicating cell content, hexosamine, indicating glycosaminoglycane content, and hydroxyproline, indicating collagen content, can be determined.

CRITICAL ASSESSMENT OF THE METHOD

The sponge implantation technique has been proven to be a versatile method which was used and modified by many investigators.

MODIFICATIONS OF THE METHOD

Boucek and Noble (1955) implanted polyvinyl sponges in rats, hamsters, rabbits and humans.

Holm-Pedersen and Zederfeldt (1971) implanted 2 cubes $10\times10\times10$ mm of cellulose sponge connected with a silk suture. After various implantation periods, the sponges were dissected free and the strength of the connection between the two parts of the sponge was determined after removal of the connecting suture.

Paulini et al. (1974, 1976) implanted polyester-polyurethane sponges which were inserted at both ends of a 15 mm long PVC tube separated by a cotton wool plug.

Bonta et al. (1979) used polyether sponges measuring $4\times1.5\times0.5$ cm. A thin polyethylene cannula is inserted into a hole of the sponge and fixed with two stitches. After implantation of the sponge the cannula is pulled through a subdermal tunnel to a neck incision where about 1.5 cm is exteriorized and closed with a tube sealer. One ml of a 2% carrageenin solution is injected into the sponge via the cannula. To study the local effect of drugs, the test compounds can be injected together with the carrageenin. The drugs can be administered repeatedly at any time.

The cannulated sponge method was further modified by Bragt et al. (1980) using a subdermally implanted Teflon cylinder. This cylinder is provided with holes to ensure contact and exchange between the inner chamber and the surrounding tissue and with two cannulae allowing injection of material and withdrawal of exudate at any given stage of granuloma development.

Damas and Remacle-Volon (1992) implanted in rats sterilized polyester sponges which were removed after 4 h and weighed.

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H.3.2.3.3 Glass rod granuloma

PURPOSE AND RATIONALE

The glass rod granuloma as first described by Vogel (1970) reflects the chronic proliferative inflammation. Of the newly formed connective tissue not only wet and dry weight, but also chemical composition and mechanical properties can be measured.

PROCEDURE

Glass rods with a diameter of 6 mm are cut to a length of 40 mm and the ends rounded off by flame melting. They are sterilized before implantation by boiling in water. Male Sprague-Dawley rats with an initial weight of 130 g are anaesthetized with ether, the back skin shaved and disinfected. From an incision in the caudal region a subcutaneous tunnel is formed in cranial direction with a closed blunted forceps. One glass rod is introduced into this tunnel finally lying on the back of the animal. The incision wound is closed by sutures. The animals are kept in separate cages. The rods remain *in situ* for 20 or 40 days. Treatment with drugs is either during the whole period or only during the last 10 or 2 days. At the end the animals are sacrificed under CO_2 anesthesia. The glass rods are prepared together with the surrounding connective tissue which forms a tube around the glass rod. By incision at one end the glass rod is extracted and the granuloma sac inverted forming a plain piece of pure connective tissue. Wet weight of the granuloma tissue is recorded. The specimens are kept in a humid chamber until further analysis. For measurement of the mechanical properties the specimens are fixed into the clamps of the Instron$^{(R)}$ instrument allowing a gauge length of 30 mm. The load until break is recorded with a crosshead speed of 50 mm/min. In order to calculate tensile strength (N/mm^2), the value of load at rupture (N) is divided by cross sectional area (measured as volume = wet weight divided by length). Finally, the granuloma tissue is dried and the dry weight is recorded. In addition, biochemical analyses, such as determination of collagen and glycosaminoglycans, can be performed.

EVALUATION

Several parameters can be determined by this method. Granuloma weight was reduced by corticosteroids depending on dose and time of administration and was also diminished after treatment with nonsteroidal anti-inflammatory agents and lathyrogenic compounds. Furthermore, antiproliferative terpenoids reduced the granuloma weight. The mechanical parameters showed different results after these drugs indicating a different mode of action. Treatment with corticosteroids increased tensile strength. Only after long term treatment with toxic doses a decrease was found. Anti-inflammatory compounds, such as acetylsalicylic acid or indomethacin and antiproliferative terpenoids showed an increase of strength at medium and high doses.

CRITICAL ASSESSMENT OF THE METHOD

In contrast to most other granuloma methods, the glass rod granuloma measures the late proliferative phase of inflammation. Since the newly formed connective tissue is not contaminated with the irritant biochemical analyses can be performed. The peculiar feature is the possibility to study the mechanical properties of newly formed proliferative connective tissue.

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H.3.3 Side effects of anti-inflammatory compounds

H.3.3.1 General considerations

Gastrointestinal side effects are among the most frequent of the untoward or adverse reactions associated with orally ingested anti-inflammatory or anti-arthritic agents. The risk of gastrointestinal ulceration, bleeding and even perforation with non-steroidal anti-inflammatory drug therapy is well known. The mechanisms by which these drugs cause gastro-intestinal irritation are complex (Rainsford 1989). Deleterious effects may result from local actions, which cause inju-

ries to the submucosal capillaries with subsequent necrosis and bleeding, or from inhibition of the formation of protective prostaglandins.

H.3.3.2 Ulcerogenic effect in rats

PURPOSE AND RATIONALE

Gastric irritation properties of orally administered compounds are evaluated in fasted rats. Following treatment the animals are sacrificed after predetermined time intervals. The stomachs are removed and inspected for irritation and ulcers.

PROCEDURE

Groups of 10 male Wistar rats with an weight between 150 and 175 g are used. They are starved 48 h (water ad libitum) prior to drug administration. The test drugs are administered orally in 10 ml/kg as aqueous solution or suspension. Doses are chosen which are highly active in the anti-inflammatory tests in rats. The animals are sacrificed 3, 5 or 7 h post drug. Control animals are sacrificed after 7 h. Stomachs are removed and placed on saline-soaked filter paper until inspection. A longitudinal incision along the greater curvature is made with fine scissors. The stomach is inverted over the index finger and the presence or absence of gastric irritation is determined. The presence of a single or of multiple lesions (erosion, ulcer or perforation) is considered to be positive. The number of ulcers and the occurrence of hyperemia is noted.

EVALUATION

The number of animals with one or more lesions of the stomach is calculated as percentage of the animals of the test group. Running various doses, an ED_{50} value can be calculated. Standard compounds which produce gastric lesions are: Acetylsalicylic acid (10–100 mg/kg); naproxene (5–50 mg/kg); indomethacin (1–10 mg/kg).

CRITICAL ASSESSMENT OF THE METHOD

A good correlation between gastro-intestinal side effects in man and the ulcerogenic effects in rats has been found. The anti-inflammatory compounds of the pyrazolone type, like phenylbutazone, are almost devoid of these effects, whereas non-steroidal anti-inflammatory compounds of the classical phenyl acetyl type show ulcerogenic properties which parallel their therapeutic effect.

MODIFICATIONS OF THE METHOD

Gastro-intestinal irritation afflicts not only the stomach but also the small intestine. These lesions are easier to detect after repeated doses than after a single administration. Therefore, the animals are treated once a day orally for 4 days. During this period, they have free access to food and water. On day 5 (24 h after the last dose being given), the animals are sacrificed and the stomachs and small intestines removed. A longitudinal incision along the lesser curvature is made. The stomach and the small intestine are rinsed in running water and the presence or absence of ulcers is determined.

A computer-planimetric analysis of the areas of lesions has been published by Szabo et al. (1985). The stomachs are formalin fixed *in situ* by oral dosing upon death and after opening are pinned on to a small cork base with mucosa upwards. The cork base and stomach are placed in a Petri dish under a stereomicroscope and illuminated from above with a cold-light source on flexible fiberoptic leads. An extension tube from the microscope projects the image from this onto an adjacent planimeter (Microplan II), which is essentially a digitized tablet with built-in microprocessor enabling data to be calculated for subsequent printout digitally. The output indicates area, number, length, or width of the lesions as totals.

In addition to evaluation of gastric lesions, Goburdhun et al. (1978) measured the decrease of intestinal tensile strength which occurred after two oral doses of indomethacin but not of aspirin.

Another refinement of the method is the assay for gastro-ulcerogenic activity of non-steroidal anti-inflammatory drugs in cholinomimetic-treated mice using visual image analysis as published by Rainsford (1987).

Alich et al. (1992) assessed the gastric mucosal damage due to aspirin and copper aspirinate by gastric mucosal potential difference changes.

Ford et al. (1995) assessed the intestinal permeability changes induced by nonsteroidal anti-inflammatory drugs in the rat. A subcutaneous air pouch was formed by injection of 10 ml sterile air prior to the experiment. Five ml of a 0.4% carrageenan solution were injected into the pouch simultaneously with a subcutaneous injection of various doses of the non-steroidal anti-inflammatory drug and the rats allowed access to food. After various time intervals, different markers were given orally and urine collected for 5 h. The use of [^{51}Cr]-EDTA as marker was found to be the most sensitive and reproducible method. The results correlated well with data of ulcer formation.

Scarpignato et al. (1995) evaluated NSAID-induced gastric mucosal damage by continuous measurement of gastric potential difference in the rat. The method allows simultaneous measurement of gastric potential difference and intragastric pH with an automatic data analysis.

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H.3.3.3 Measurement of gastric mucosal damage by intragastric inulin

PURPOSE AND RATIONALE

Wittmers et al. (1990) used intragastric inulin as a measure of mucosal damage caused by aspirin. The movement of inulin out of the gastric contents into the stomach wall and into the vascular compartment is an estimate of mucosal damage.

PROCEDURE

Adult male Sprague-Dawley rats are anesthetized with sodium pentobarbital and functionally nephrectomized by placing a ligature around both renal pedicles in order to prevent loss of inulin by renal clearance. One cannula is inserted in the carotid artery, another into the stomach. The duodenum is ligated 1 cm distal to the gastro-duodenal junction. The stomach is rinsed with saline and then the test solutions (total volume 2 ml/100 g body weight) are given intragastrally. The control solution contains 0.01 g/100 ml inulin and 0.30 μCi/ml ^{3}H-inulin. Test solutions contain the irritant, e.g. 100, 200, or 500 mg/kg aspirin. During the exposure period of 90 min blood is withdrawn in 15 min intervals. At the end of the exposure period a sample of gastric contents is obtained, the esophagus ligated at its junction with the stomach, and the animal sacrificed. The stomach is removed, opened along the lesser curvature, rinsed with saline, and pinned across a rubber stopper. Ten full thickness tissue samples are removed with a No. 2 cork bore in a predetermined pattern. The samples of tissue, plasma, and gastric content are digested and bleached with 0.2 ml 60% perchloric acid and 0.5 ml 30% hydrogen peroxide at a temperature of 80 °C for 20–30 min. Ten ml of liquid scintillation cocktail is added and ^{3}H-inulin is determined by liquid scintillation counting.

EVALUATION

The ^{3}H-inulin radioactivity is expressed as disintegrations per min per ml of plasma or per gram of tissue. Plasma ^{3}H-Inulin levels being dose-dependent increased after treatment are plotted versus time in comparison with control. ^{3}H-Inulin content in the gastric wall is dose-dependent increased after treatment especially in the antrum region.

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H.3.3.4 Determination of blood loss

PURPOSE AND RATIONALE

Use of non-steroidal anti-inflammatory drugs induces micro bleeding which is not detected by observation of overt ulcers. Moreover, determination of the amount of lost blood may give a better quantitative assessment of gastrointestinal side effects.

PROCEDURE

Male Sprague-Dawley rats with an weight of 180–200 g are used. They are fasted in wire-floor cages for 24 h with free access to water. The animals receive the test drug or the standard compound (300 mg/kg acetylsalicylic acid) by gavage in 1 ml of 1% Arabic gum. For determination of fecal blood loss, the rats are kept in individual metabolism cages and food is given to them 5 h after treatment. Feces from each rat are collected separately every 24 h for 5 days. Heme is extracted from the feces and determined spectrophotometrically. For determination of the site of gastrointestinal bleeding the rats are sacrificed 5 h after treatment by ether inha-

lation. The stomach is tied on both the esophageal and pyloric ends, removed and washed free of external blood. The stomach content is released into a graduated test tube. Then the stomach is rinsed with 5 ml of saline. Similarly, the intestine is cut into 3 portions, each being tied at both ends. The contents after flushing the intestines twice with saline are collected. In the fluid of stomach and intestine content heme is determined spectrophotometrically.

EVALUATION
Time course of blood loss in the feces and dose-depended increase of blood in the content of stomach and intestine are determined.

CRITICAL ASSESSMENT OF THE METHOD
The determination of the time course of blood loss and the site of bleeding is interesting for in depth investigations but has to be regarded as a secondary screen.

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H.4 Antipyretic activity

H.4.0.1 General considerations

Treatment with antipyretics has been very important in the pre-antibiotic era. Nevertheless, for treatment of acute viral diseases and for treatment of protozoal infections like malaria reduction of elevated body temperature by antipyretics is still necessary. For anti-inflammatory compounds, an antipyretic activity is regarded as a positive side effect. To evaluate these properties, fever is induced in rabbits or rats by injection of lipopolysaccharides or Brewer's yeast.

H.4.0.2 Antipyretic testing in rats

PURPOSE AND RATIONALE
The subcutaneous injection of Brewer's yeast suspension is known to produce fever in rats. A decrease in temperature can be achieved by administration of compounds with antipyretic activity.

PROCEDURE
A 15% suspension of Brewer's yeast in 0.9% saline is prepared. Groups of 6 male or female Wistar rats with a body weight of 150 g are used. By insertion of a thermocouple to a depth of 2 cm into the rectum the initial rectal temperatures are recorded. The animals are fevered by injection of 10 ml/kg of Brewer's yeast suspension subcutaneously in the back below the nape of the neck. The site of injection is massaged in order to spread the suspension beneath the skin. The room temperature is kept at 22–24 °C. Immediately after yeast administration, food is withdrawn. 18 h post challenge, the rise in rectal temperature is recorded. The measurement is repeated after 30 min. Only animals with a body temperature of at least 38 °C are taken into the test. The animals receive the test compound or the standard drug by oral administration. Rectal temperatures are recorded again 30, 60, 120 and 180 min post dosing.

EVALUATION
The differences between the actual values and the starting values are registered for each time interval. The maximum reduction in rectal temperature in comparison to the control group is calculated. The results are compared with the effect of standard drugs, e.g. aminophenazone 100 mg/kg p.o. or phenacetin 100 mg/kg p.o.

CRITICAL ASSESSMENT OF THE METHOD
The antipyresis test in rats in can be regarded as a classical method in pharmacology.

MODIFICATIONS OF THE METHOD
Stitt and Shimada (1991), Shimada et al. (1994) induced fever in rats by microinjecting 20 ng PGE_1 directly into one of the brain's circumventricular organs of the rat known as the organum vasculosum laminae terminalis.

Luheshi et al. (1996) induced fever by intraperitoneal injection of 100 µg/kg lipopolysaccharide into rats and measured the inhibition of fever by interleukin-1 receptor antagonist.

Telemetry has been used to record body temperature in animals (Riley et al. 1978; Gallaher et al. 1985; Clement et al. 1989; Guillet et al. 1990; Kluger et al. 1990; Bejanian 1991; Watkinson et al. 1996; Miller et al. 1997).

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H.4.0.3 Antipyretic testing in rabbits

PURPOSE AND RATIONALE

Lipopolysaccharides from Gram-negative bacteria, e.g. *E. coli*, induce fever in rabbits after intravenous injection. Only lipopolysaccharide fractions are suitable, which cause after 60 min an increase of body temperature of 1 °C or more at a dose between 0.1 and 0.2 µg/kg. In the rabbit, two maxima of temperature increases are observed. The first maximum occurs after 70 min, the second after 3 h.

PROCEDURE

Rabbits of both sexes and of various strains with a body weight between 3 and 5 kg can be used. The animals are placed into suitable cages and thermocouples connected with an automatic recorder are introduced into the rectum. The animals are allowed to adapt to the cages for 60 min. Then 0.2 ml/kg containing 0.2 µg lipopolysaccharide are injected intravenously into the rabbit ear. Sixty min later the test compound is administered either subcutaneously or orally. Body temperature is monitored for at least 3 h.

EVALUATION

A decrease of body temperature for at least 0.5 °C for more than 30 min as compared with the temperature value before administration of the test compound is regarded as positive effect. This result has been found after 45 mg/kg phenylbutazone s.c. or 2.5 mg/kg indomethacin s.c.

CRITICAL ASSESSMENT OF THE METHOD

Measurement of body temperature in rabbits with polysaccharide induced fever is a more sensitive test than the yeast fever in rats. Furthermore, the method is used as a decisive test for the absence of pyrogens in parenteral drugs by several pharmacopoeias such as USP 23 (1955).

MODIFICATIONS OF THE METHOD

Cashin and Heading (1968) described a simple and reliable assay for antipyretic drugs in **mice**, using intracerebral injection of pyrogens.

Davidson et al. (1991) tested the effect of human recombinant lipocortin on the pyrogenic action of the synthetic polyribonucleotide polyinosini: polycytidylic acid in rabbits.

Yeast-induced pyrexia in **rats** has been used for antipyretic efficacy testing by Loux et al. (1982) and Cashin et al. (1977).

van Miert et al. (1977) studied the effects of antipyretic agents on fever and ruminal stasis induced by endotoxins in **conscious goats**.

Petrova et al. (1978) used turpentine-induced fever in rabbits to study antipyretic effects of dipyrone and acetylsalicylic acid.

Lee et al. (1985) studied the antipyretic effect of dipyrone on endotoxin fever of **macaque monkeys**.

Loza Garcia et al. (1993) studied the potentiation of chlorpromazine-induced hypothermia by the antipyretic drug dipyrone in anesthetized rats.

Shimada et al. (1994) studied the mechanism of action of the mild analgesic dipyrone preventing fever induced by injection of prostaglandin E_1 or interleukin-1β into the organum vasculosum terminalis of rat brain.

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Chapter I
Antiarthrotic and immunmodulatory activity[1]

I.1
Anti-arthrotic activity[2]

I.1.0.1
General considerations

Multifactorial causes can lead to osteoarthritis (OA), and its pathogenesis is not clearly understood as yet. The main characteristics of OA are the slowly progressing deterioration of the articular cartilage, accom-

[1] Reviewed and amended by R. Schleyerbach, with contributions to the first edition by R. X. Raiss and R. R. Bartlett.

[2] Contributions in the first edition by R. X. Raiss.

panied by intermitted painful inflammatory episodes, and a continuous subchondral bone remodeling, often resulting in osteophyte formation in non-weight-bearing joint areas. Because of the lack of innervation and vascularization of cartilage, the destruction of this specific tissue remains unnoticed until other joint compartments are involved such as synovial membranes, answering with reactive synovitis to cartilage debris, or mechanoreception changes in the underlying bone, or until the diminution of articular cartilage results in a radiographically detectable joint space narrowing.

Therefore, analgesic and anti-inflammatory therapy has been the major treatment for OA in the past, and NSAIDs still constitute 92% of the drugs used against OA. Intraarticular injections of corticosteroids are also applied, although with geographically variable emphasis. Nevertheless they are still considered a useful tool in severe cases. Both classes of drugs are now reviewed more carefully with regard to their potentially harmful effects on cartilage maintenance and chondrocyte function – which leads to a more critical approach in drug selection. So-called 'chondroprotective' drugs, mainly sulfated polysaccharides, played a certain role in the pharmacotherapy of OA in Central Europe and some Eastern countries, but failed to demonstrate clinical efficacy, and now have lost significance.

Anti-oxidative enzymes or drugs such as superoxide dismutase or diacerein are also considered to influence osteoarthritic conditions. Recently, matrix metalloprotease-inhibitors (MMP-I), originally designed to inhibit tumor cell invasion, have shown promising results in counteracting the progressive enzymatic cartilage degradation, and some compounds are being developed for this indication. A further treatment gaining interest are derivatives of hyaluronic acid, which are applied intraarticularly in a series of injections. Their mechanism, however, is not clear yet, and most preparations have been filed as 'devices' rather than 'drugs', claiming a viscosupplementation with anti-inflammatory, analgesic, and chondroprotective properties.

I.1.1 *In vitro* methods for anti-osteoarthritic activity

I.1.1.1 General considerations

Since most of the drugs in use for OA were originally selected for other (e.g. arthritic) indications and only subsequently claimed to be effective in OA, they have not been primarily selected and optimized by *in vitro* assays specific for this condition. Thus, the indication lacks commonly agreed upon *in vitro* assays as well as clearly defined standard drugs to evaluate such models. Correspondingly, the variety of assay systems used to test compounds for their effect on cartilage maintenance and/or degradation is large, and the list below reflects this multitude.

The *in vitro* systems applied to assess drug effects upon chondrocytes range from homogenates of cartilage (Yu et al. 1991; Vignon et al. 1991; Zafarullah et al. 1992) over chondrocyte monolayers at different culture conditions and passages, suspensions of aggregated chondrocytes or clusters, cells cultured in or over an artificial matrix like agarose to cartilage explants and even organ cultures (Korver et al. 1989) like mouse patellas (Verschure et al. 1994). The species used in these studies vary over an equally wide range from mouse (Mohamed-Ali 1992), rat (Ismaiel et al. 1991; Seed et al. 1993; Seong et al. 1994; Srinivas et al. 1994), rabbit (Akatsuka et al. 1993; Collier, Ghosh 1991; Shimazu et al. 1993), dog (Venn et al. 1990), cattle, to human tissues (Bulstra et al. 1992; Green et al. 1995) derived from normal as well as osteoarthritic conditions. The chondrocyte and cartilage explant culture systems used for several years are described in more detail below with special emphasis on comparability and standardization.

PURPOSE AND RATIONALE

Articular chondrocytes not only control the regular balance of matrix synthesis and degradation in healthy cartilage turnover, but are also regarded as key players in the enhanced degradation and finally reduced synthesis of matrix components in pathological conditions like OA. The two main cartilage constituents are collagen type II fibrillar network, and proteoglycans attached to hyaluronic acid filaments, also termed aggrecans. The latter are the more sensitive and the first ones to change in cartilage degradation. Therefore, the *in vitro* assays are performed mainly with chondrocytes, and the parameters measured focus on proteogly can synthesis and/or degradation.

The exact mechanisms of cartilage pathophysiology are not yet elucidated, but enzymatic degradation involving metallo-proteinases are considered the main events. *In vitro*, interleukin-1 and retinoic acid induce enhanced matrix degradation as well as reduced matrix synthesis, as observed in OA. Their role in the actual disease process *in vivo*, however, remains obscure. They therefore are used rather as tools to induce a disease-relevant condition *in vitro* than being subject of direct pharmacological intervention. Thus, the *in vitro* assays described here are suitable to compare and select a variety of drugs for their effect upon the main biological activity of articular chondrocytes. To address the respective mechanisms of drug action, more spe-

cific follow-up assays like enzyme inhibition or cytokine release or inhibition tests should be applied.

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I.1.1.2 Modulation of cellular proteoglycan metabolism

PURPOSE AND RATIONALE

In primary cultures, articular chondrocytes grown in an artificial matrix after digestion of the original bone, maintain their characteristic synthesis and turnover rate of cartilage matrix macromolecules for a long time. These metabolic processes can be influenced pharmacologically. In the following assay, compounds are tested for their effect upon the normal turnover of cartilage matrix by chondrocytes. The test is used to detect stimulation of matrix formation, but also to check for potential impairment of cartilage function. Specific matrix staining reveals the amount of newly formed matrix remaining around the cells at the end of treatment. Alternatively, incorporation of radiolabeled sulfate into the newly formed proteoglycans allows to quantitate the anabolic activity at the end of the experiment.

PROCEDURE

Reagents

A 1% (w/v) solution of Pronase from Boehringer in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10% FCS.

A 0.025% (w/v) solution of Collagenase type II, activity 242 U/mg, from Worthington, corresponding to an activity of 6 U/ml in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10% FCS.

Hank's balanced salt solution (HBSS) is obtained from Biochrome.

Ham's F12 is supplemented with 50 µg/ml gentamycine and 2.5 µg/ml amphotericin B.

A sterile stock solution of 5 mg/ml ascorbic acid in Ham's F12 is prepared, and aliquots are stored at –20 °C.

A 2% solution of low melting agarose from Seaplaque in 0.9% NaCl is prepared by heating in a microwave, and stored in a water bath at about 50 °C.

A buffer of 25 mM sodium-acetate (2.051 g/l) with 0.4 M magnesium-chloride 6-hydrate (81.32 g/l) is prepared, and adjusted with acetic acid to pH 5.6.

A staining solution is prepared with 0.1 g of alcian-blue, obtained from Sigma, in 67.5 ml buffer, filtered, and supplemented with 10 ml of a 25% solution of glutaraldehyde.

An 8 M guanidinium-hydrochloride solution is prepared.

Tissue and cell preparation

Fetlocks of freshly slaughtered steers (age 18 to 20 months) are skinned and the metacarpo-phalangeal joint opened under semi-sterile conditions. With a sterile scalpel, articular cartilage is then carefully removed from the underlying bone from all accessible cartilaginous regions and transferred into a sterile Ham's F12 solution at +4 °C. The tissue is washed with Ham's F12 to remove adherent synovial fluid. The pieces are then transferred into a 150 ml trypsinizing flask, containing the pronase solution including the added serum, and incubated with gentle stirring for 1 h at 37 °C and 95% humidity. The fluid is then removed, and the collagenase solution including the added serum is incubated with gentle stirring overnight. The resulting cell suspension is first filtered through a 90 µm and then a 50 µm Nylon filter and then centrifuged at 800 rpm for 10 min. Resuspension and washing is performed with HBSS and cells are counted and checked for vitality under the microscope using the Eosin staining. The vitality level should reach at least 95%. A cell suspension is prepared of 4×10^6 cells/ml Ham's F12 supplemented with 20% FCS.

To prepare the agarose cell cultures, 24 well plates are coated with 0.2 ml/well of a 1 : 1 mixture of the 2% agarose solution with preheated Ham's F12 and left at room temperature to gel. Then 0.1 ml/well (0.2 ml/well for radiolabeling) of a 1 : 1 mixture of the above described cell suspension with the 2% agarose solution is added. Care has to be taken to maintain an even cell suspension, and not to overheat the cells during this procedure. After gel formation at room temperature, the multiwell plates are placed in the incubator, and 0.5 ml/well medium is added either 4 h later or the following day. The medium consists of Ham's F12 supplemented with 5% FCS and 25 µg/ml ascorbic acid, and is changed every second day.

Assay

The assay starts 5 days after cell preparation. Compounds are added to the medium in a final concentration of 10 µM with 6 to 8 replicae per compound, and added anew with each change of medium over a total period of 8 days. The concentration can be varied according to the expected potency of the drug studied. An untreated control group as well as standard compound groups are always included. As standard compound, pentosane polysulfate to check for matrix increase, or retinoic acid to cause matrix decrease, can be applied.

At the end of treatment, the medium is removed, the wells washed 3× with 500 µl of medium without supplements, and 1 ml/well of the staining solution is added for 48 h. After removal of the supernatant, the following washing steps are performed for 10 min each:

- 3×500 µl/well 3% acetic acid,
- 1×500 µl/well3% acetic acid in 25% ethanol,
- 2×500 µl/well 50% ethanol,
- 1×500 µl/well70% ethanol.

With 500 µl/well of 8 M guanidinium hydrochloride solution, the bound stain is then extracted for 24 h at +4 °C. After shaking the plates gently for 10 min, 100 µl/well of each supernatant is then transferred to round-bottom microtiter-plates, and the extinction photometrically assessed in the plate-reader at a wavelength of 610 nm.

EVALUATION

The extinction is expressed in percentage as staining density with the control values defined as 100%. Values ≥ 110% are interpreted as stimulation of matrix formation, values lower than 80% as inhibition of matrix formation. Experiments with 8 wells/treatment usually exhibit a standard deviation below 7%.

CRITICAL ASSESSMENT OF THE TEST

The described method is suitable to compare up to 50 drugs in one experiment. The price for this is the limited quantification, as the staining is not strictly stoichiometric, and does not allow the distinction between matrix synthesis and degradation. For more detailed assessment, radiolabeling is the better choice. The limitation of these primary culture assays lies in the elaborate preparation and isolation of the chondrocytes. Several attempts to immortalize this differentiated mesenchymal cell type have resulted in the loss of cartilage-specific properties. A new cell line developed by MB Goldring (Green et al. 1995) might overcome this difficulty, but has not yet been reported to be modified pharmacologically.

MODIFICATIONS OF THE TEST

The agarose culture system for chondrocytes, originally described by Benya and Schaffer (1982), has been well characterized by Aydelotte et al. (1988, 1992), and the effects of different agarose densities have been studied by Verbruggen et al. (1990).

Instead of agarose gel cultures, some authors use 3D chondrocyte clusters in suspension (Bassleer et al. 1990, 1992; Henrotin et al. 1992), or suspensions over agarose (Archer et al. 1990), or embedded in collagen gels (Malemud et al. 1994).

Alternatively, encapsulation in alginate beads, either directly after isolation (Guo et al. 1989), even as primary culture for several months (Häuselmann et al. 1994), or after expansion in monolayers (Bonaventure et al. 1994), offers the opportunity to recover the chondrocytes later by depolymerization of the alginate.

Monolayers of articular chondrocytes can be used as well, but preferentially short-term (up to three days of culture), as under this culture condition chondrocytes tend to dedifferentiate to a fibroblast-like appearance and metabolic program. Authors using this modification are e.g. Kolibas, Goldberg (1989), Lane et al. (1992), and McCollum et al. (1991). The importance of culture conditions is addressed in the study by Seid et al. (1993), and that of culture duration in the paper by van der Kraan et al. (1992).

A dot blot assay by cuprolinic blue precipitation has been described by Jortikka et al. (1993), which is restricted to serum free conditions. Instead of matrix staining, radiolabeling can be applied as described in the next assay. In this case, the amount of cells should be doubled per well to assure sufficient label incorporation.

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I.1.1.3 Cellular chondrocytic chondrolysis

PURPOSE AND RATIONALE

In this assay a disease-relevant situation is achieved by adding interleukin-1 (IL-1) to articular chondrocytes grown in agarose gel. IL-1 suppresses proteoglycan (PG) synthesis as well as increases their degradation, and thus results in a process which is also observed in degradative joint diseases *in vivo*. This process is termed chondrocytic chondrolysis. The test is used to detect the potential interference of a drug with this pathological process. Effect upon PG synthesis is studied by radiolabeling at the end of the experiment, and measuring the amount of incorporated sulfate. The effect upon PG degradation, and its release from the cellular environment, can be examined by prelabeling with $Na_2{}^{35}SO_4$, and following the amount of released incorporated sulfate over time from the supernatant with each or every second medium change.

PROCEDURE

Reagents

A 1% (w/v) solution of Pronase from Boehringer in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10% FCS.

A 0.025% (w/v) solution of Collagenase type II, activity 242U/mg, from Worthington, corresponding to an activity of 6 U/ml in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10% FCS.

Hank's balanced salt solution (HBSS) is obtained from Biochrome.

Ham's F12 is supplemented with 50 µg/ml gentamycin and 2.5 µg/ml amphotericin B.

A sterile stock solution of 5 mg/ml ascorbic acid in Ham's F12 is prepared, and aliquots are stored at –20 °C.

A 2% solution of low melting Agarose from Seaplaque in 0.9% NaCl is prepared under heating and microwave application, and stored in a water bath at about 50 °C.

Human recombinant interleukin-1 (IL-1)α or β from Genzyme is stored in aliquots at –20 °C.

For radiolabeling, $Na_2{}^{35}SO_4$ is purchased from Amersham.

A 4 M and 8 M guanidinium-hydrochloride (GuHCl) solution is prepared.

Tissue and cell preparation

Tissue and cell preparations are performed as for the previous assay, except the initial number of cells/well should be 400 000, corresponding to a cell-containing gel volume of 0.2 ml/well, and the medium added is supplemented with 10% FCS and 25 µg/ml ascorbic acid. It is changed every second day.

Assay

The assay starts 6 days after cell preparation. Except for a control group, interleukin-1α is added in a concentration of 3 U/well, and added anew with each consecutive medium change. Except for the IL-1-control group, compounds are added to the medium in a final concentration of 10 µM with 4 replicae per compound, and with the subsequent medium changes. The concentration can be varied according to the expected potency of the drug studied. At the end of an 8 day treatment, the medium is replaced by medium containing 1 µCi $Na_2{}^{35}SO_4$/well and incubated for 24 h. The supernatant is then removed, mixed 1 : 1 with 8 M GuHCl, and separated with a PD10-Sephadex column into free versus incorporated sulfate. The multiwell plates with the remaining gels are deep frozen for at least 24 h to facilitate solubilization, thawed and then extracted with 500 µl/well 8 M GuHCl supplemented with inhibitors. The content of each well is then centrifuged at 13 000 rpm for 30 min. After this step, the supernatant contains the matrix trapped around the cells. This is equally separated by a PD10 Sephadex column into free versus incorporated radiolabel. The probes containing the incorporated sulfate and derived from both fractions (medium and gel) are then mixed with scintillating fluid and assessed in a β-scintillation counter.

EVALUATION

Counts per minute (cpm) from medium and gel fraction are calculated and related to the total well content. They are added if total incorporation is measured, or left separately, in case the ratio between released versus retained label is of interest. The data are converted into percent incorporation, with the values of the untreated control group or those of the IL-1 control group serving as 100%.

CRITICAL ASSESSMENT OF THE TEST

This is a sensitive test, in which an adequate labeling protocol can provide detailed information. The time- and material-consuming separation of free from incorporated radiolabel at the end, however, limits the size of the experiments and number of compounds to be studied.

In both cellular tests drug effects should be checked for (anti)proliferative activity in a separate proliferation test.

MODIFICATIONS OF THE TEST

If the catabolic response to a drug is of more interest than the anabolic one, the radiolabeling can be shifted to a time point prior to treatment, and the release of incorporated sulfate into the supernatant will allow to follow the time course and amount of PG degradation. This prelabeling should not start earlier than 4 days after plating to assure a comparable matrix synthesis rate. Because IL-1 suppresses PG synthesis at a lower concentration than it stimulates its degradation, a double to triple amount of IL-1 should be used in this modification.

Instead of bovine, human chondrocytes can be used (Raiss et al. 1992). One should allow 4 days of adjustment in the agarose system before starting treatment with human cells. If available, human serum gives a higher PG synthesis rate than fetal calf serum (Oestensen et al. 1991). The heterogeneity of responses depends on the individual source and should be considered (Verbruggen et al. 1989).

When using human instead of bovine cells, the stimulation of degradation and inhibition of synthesis is more effective with IL-1β than IL-1α: a concentration of 0.1 U/ml results in a reduction of PG synthesis of ca 50% (Raiss et al. 1995). When incubating with radiolabeled sulfate, the exposure time should be doubled to 48 h to yield sufficient incorporation.

To exclude direct interference of a drug with interleukin-1, all-trans retinoic acid can be used instead of this cytokine.

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I.1.1.4 Cartilage explant chondrolysis

PURPOSE AND RATIONALE

Chondrocytes vary in their metabolic activity and cytokine response depends on the relative location within the joint (surfacial vs deep, weight-bearing vs non-weight-bearing, etc). Therefore, the cellular assays are a homogeneous mixture of an otherwise heterogeneous cell population. Two reasons suggest a verification of the cellular results obtained in tissue culture assays: First, chondrocytes are more reactive after isolation compared to those in tissue culture, which may lead to false positive results. Second, intact cartilage matrix acts as barrier for certain compounds of high molecular size and fixed charge, so that they may not reach their target cells. Therefore, explant assays are recommended as a follow-up to the cellular tests.

PROCEDURE

Reagents

Ham's F12 medium is supplemented with 50 µg/ml gentamycin and 2.5 µg/ml amphotericin B.

A sterile stock solution of 5 mg/ml ascorbic acid in Ham's F12 is prepared, and aliquots are stored at –20 °C.

Human recombinant interleukin-1 (IL-1)α or β from Genzyme is stored in aliquots at –20 °C.

For radiolabeling, $Na_2{}^{35}SO_4$ is purchased from Amersham.

A 4 M and 8 M guanidinium-hydrochloride (GuHCl) solution is prepared.

Tissue preparation

Fetlocks of freshly slaughtered steers (age 18 to 20 months) are skinned and the metacarpo-phalangeal joint opened under semi-sterile conditions. With a sterile punch (as used for obtaining skin biopsies) full thickness disks of cartilage are obtained from all accessible cartilaginous areas and their wet weight is assessed. In each well, 1 ml of medium is added, consisting of Ham's F12 supplemented with 10% FCS and 25 µg/ml ascorbic acid, and approximately 30 mg wet weight of cartilage are transferred corresponding to 3 discs of 4 mm diameter.

Assay

The assay is started 1 to 2 days after tissue preparation. Except for a control group, interleukin-1α is added in a concentration of 8 U/well, and with each of the following medium changes (every second day). Except for an IL-1-control group, compounds are added to the medium in a final concentration of 10 µM with 6 to 8 replicae per compound, which are also added with each medium change. The concentration can be varied according to the expected potency of the drug studied. At the end of an 8 day treatment, the medium is replaced by a medium containing 15 µCi $Na_2{}^{35}SO_4$/well and incubated for 24 h. The supernatant is removed, mixed 1 : 1 with 8 M GuHCl, and separated with a PD10-Sephadex column into free versus incorporated sulfate. The explants are washed three times with Ham's F12 at +4 °C, and extracted with 1 ml/well 4 M GuHCl supplemented with inhibitors for 48 h, and then a second time with 0.5 ml/well for 24 h. Both fractions are mixed, and separated with a PD10 Sephadex column into free versus incorporated sulfate. The samples containing the incorporated sulfate derived from medium as well as explant extraction are then mixed with scintillating fluid and assessed in a β-scintillation counter.

EVALUATION

Counts per minute (cpm) from medium and explant fractions are calculated related to mg wet weight of cartilage of the respective wells. They are either added if total incorporation is measured, or left separately, in case the ratio between released versus retained matrix is of interest. The data are converted into percent incorporation in comparison with the values of the untreated control or of the IL-1 control group serving as 100%.

CRITICAL ASSESSMENT OF THE TEST

Punched discs of similar size standardize the surface/volume ratio, and give more reproducible results than chips of cartilage obtained by scalpel dissection. A disadvantage is the greater amount of cartilage needed, which makes it unsuitable for human tissue obtained from joint replacement surgery. When using human

tissue, interpretation and comparison of results should be restricted to the same source: striking differences occur between specimens from surgery or postmortem, between different joints (hip vs knee), and different ages of the donor (young or adolescent vs 35 years and older), as well as different stages of severity and the duration of degenerative joint diseases in cases of surgical specimens.

MODIFICATIONS OF THE TEST

The effect of serum concentrations on proteoglycan synthesis has been studied by McQuillen et al. (1986), and the effect of different concentrations of DMSO and glycerol, of importance for cryopreservation, has been examined on human fetal hip cartilage by Yang and Zhang (1991).

Some authors (Nixon et al. 1991) use bovine nasal septum as cartilage source, but the convenient homogeneity and mass of this tissue is outweighed by a matrix composition and biomechanical properties clearly distinct from articular cartilage.

Several authors use human cartilage from joint replacement surgery (e.g. Pelletier et al. 1989; Pelletier, Martel-Pelletier 1989), and some compare drug effects upon visually normal cartilage to those with fibrillated or osteoarthritic cartilage (Lafeber et al. 1992, 1993; Verbruggen et al. 1989, 1990).

A step towards organ culture represents the culture of full thickness cartilage with subchondral bone, cultured for 24 h on moist lens tissue (Chayen et al. 1994).

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I.1.2 *In vivo* methods for anti-osteoarthritic activity

I.1.2.1 General considerations

The current availability of animal models of osteoarthritis (OA) for pharmacological assessment is impeded in several aspects: Firstly, the difficulty to address a generally slow progression of cartilage destruction and deterioration of joint function as encountered in human OA with an animal model achieving sufficient similarity to human pathology in an acceptable time frame. Secondly, the discrepancy between the need of mild, reversible pathological changes, which can be modified therapeutically, and the paucity of reliable parameters with which to determine normal versus disease stages with a gradable range large enough to assess drug effects. This includes also the lack of validation (clinically as well as in animal models) of noninvasive methods to assess disease progression. Thirdly, the lack of a true disease-modifying standard drug with which to validate the pharmacological effects in respect to the predicted clinical outcome.

This results in a situation that animal models closer to human pathology like the spontaneous OA in the Hartley strain of guinea pigs or the surgically inflicted joint instability in the Pond-Nuki dog model are too elaborate to be used routinely for drug selection. On the other hand, models like the chymopapain-induced cartilage degradation in rabbits, are suitable to study drugs, but are limited in their predictive value.

It should be noted that differences exist in the pathomechanisms of cartilage destruction between rheumatoid arthritis (RA) and OA. The destruction occurring in RA is closely linked to the inflammatory process, synovial tissue proliferation and transgression across the cartilage surface, degrading cartilage proteoglycans and collagens simultaneously. In OA inflammation is only an intermitted event, not instrumental in the degenerative cartilage destruction, in which proteoglycan degradation is the early event, and collagen loss occurs at a distinctly later stage. Therefore, animal models with a predominant inflammatory component as the air pouch model or other arthritis models, even

those focusing on cartilage destruction, are not discussed in this chapter (see Sect. H.3). They are described in detail by Greenwald and Diamond (1988) and recently reviewed by Greenwald (1991, 1993).

PURPOSE AND RATIONALE

As a multitude of different events can lead to OA, equally different techniques have been used to initiate osteoarthritic conditions in animal models: Surgical methods are used to either stiffen the joint in a defined position (Palmoski, Brandt 1982; Konttinen et al. 1990; Meyer-Carrive and Ghosh 1992) or inflict joint instability by partial meniscectomy or anterior crucial ligament (ACL) dissection. They are performed mainly in dogs, rabbits (Colombo et al. 1983; Moskowitz et al. 1973, 1979), and guinea pigs (Schwartz 1985; Meacock et al. 1990). Chemical modifications like intraarticular injections of iodoacetate, cytokines like IL-1, or enzymes like chymopapain or stromelysin, are carried out mainly in rabbits (Williams et al. 1992) and chicken (Kalbhen 1983, 1987). Mechanical forces are applied on bent or opened joints like impulse loading on sheep (Lindenhayn et al. 1984) or rabbit (Farkas et al. 1987; Mazière et al. 1984) knees, resulting in trauma models of OA. Spontaneously occurring OA is described in horses (Todhunter and Lust 1992; Haakenstad 1969), some breeds of dogs (Lust et al. 1985), rhesus macaques (Pritzker et al. 1989), guinea pigs (Bendele, Hulman 1988) and several strains of mice. For pharmacological purposes only guinea pigs and STR/1N (Walton 1965; Raiss et al. 1992), STR/ORT (Dunham et al. 1989), and C57 black (Pataki et al. 1990) mice have been adapted. In all models mentioned (except horses), the relevant joint is the knee.

Since there exist recent extensive and critical reviews of OA models (Burton-Wurster et al. 1993; Pritzker 1994; Moskowitz 1990, 1992; Adams and Billingham 1982; Oegema et al. 1999), also with respect to reversibility (Pita et al. 1986), with special emphasis on drug testing (Hess, Herman 1986), and in perspective to cartilage research and markers (Malemud 1993; Carney 1991), only some representative models of each category are described here.

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I.1.2.2 Canine anterior cruciate ligament (ACL) transection model

PURPOSE AND RATIONALE

Similar to human ACL ruptures, ACL transection in the dog knee and the subsequent joint instability results in progressive cartilage erosion, fibrillation, and formation of osteophytes. This elaborate model is well characterized, and regarded of high predictive value. It is mostly known as the "Pond-Nuki dog model". These authors achieved the ACL transection originally through a lateral stab incision, whereas others also performed the ligament transection after opening the joint. Both versions lead to similar morphological and biochemical changes, and are described and illustrated in detail by Adams and Pelletier (1988).

PROCEDURE

Animals

Mongrels, beagles, greyhounds, and foxhounds are reported to be suitable, provided purebred strains are used. In general, younger animals display more repair phenomena, whereas older ones seem to show more rapid degeneration. As the epiphyses fuse at the age of 13 months, and as pronounced changes require up to 4 months to fully develop, careful planning ahead is essential.

Operation

The dogs are initially anesthetized with 30 mg/kg sodium pentobarbital i.v., followed by a continuous inhalation of 1% halothane with 1 l/min N_2O and 2 l/min O_2. After shaving and sterilizing the knee joint externally, it is fixed in a bent position at 90°, and a scalpel blade is inserted medially deep into the joint space diagonally posterior to the ACL, and parallel to the lateral border of the patellar ligament. By rotation of the blade, the ACL is then dissected, the blade withdrawn, and the wound closed. In the contralateral knee, a sham operation is performed to inflict similar disturbance to the joint tissue, but without harming the ACL. The ACL dissection results after 8 to 12 weeks, in contrast to the sham-operated contralateral knee, in fibrillation and erosion of the cartilage, more pronounced on the tibial plateau than on the femoral condyles. Also observed histologically is a loss of metachromatic staining, and a fissured surface with cell clones appears. A marked osteophytosis and subchondral sclerosis has also developed at that time. Proteoglycan content and overall cartilage thickness, however, seems to remain stable (Pelletier and Martel-Pelletier 1985) or even increase not only for several months (McDevitt et al. 1977; Vignon et al. 1983; Brandt and Adams 1989), but for up to 3 years after transection, as Brandt, Myers et al. (1991) could show. At later stages, however, severe cartilage thinning and loss is recorded (Brandt, Braunstein et al. 1991).

EVALUATION

Macroscopic inspection of cartilage and osteophytes are recorded. Histological grading based on the Mankin score has been reported to be modified by drug treatment over 7 weeks (Abatangelo et al. 1989; Schiavinato et al. 1989). As levels and activity of neutral matrix metalloproteases are elevated in cartilage and synovium (Pelletier, Martel-Pelletier 1985), they might be additional parameters of interest to profile the test compounds.

CRITICAL ASSESSMENT OF THE TEST

In this instability model, a polysulfated glycosaminoglycan preparation (Arteparon®), as well as intra-articular injections of a hyaluronic acid preparation induced some morphological and biochemical changes, whereas low-dose prednisone had no effect. As there seems to prevail an anabolic response to the instability in the articular cartilage for quite a long time, the selected time points and parameters to assess disease

progression and therapeutic success require careful consideration.

MODIFICATIONS OF THE TEST

Caron et al. (1996), Pelletier et al. (1997) investigated the *in vivo* effect of the recombinant human interleukin-1 receptor antagonist on the development of lesions in the anterior cruciate ligament transection model in dogs.

ACL transection can be performed also in an arthrotomy operation as described by Adams and Pelletier (1988), and an instability can be achieved equally by meniscectomy (Hannan et al. 1987).

The **rabbit** is the main other species used for instability models, as described in detail for partial medial meniscectomy by DiPasquale et al. (1988), and for partial lateral meniscectomy in connection with ligament transection by Colombo (1988).

Obara et al. (1993) induced osteoarthritis by surgical dissection of the anterior cruciate ligament in rabbits and investigated fluorescence distribution after intra-articular administration of fluorescein-labeled sodium hyaluronate.

A partial medial meniscectomy with ligament transection is described for the **guinea pig** by Schwartz (1988).

Layton et al. (1987) produced biomechanical stress-induced hip osteoarthritis in guinea pigs by extra-articular myectomy and tendotomy.

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I.1.2.3 Chymopapain-induced cartilage degeneration in the rabbit

PURPOSE AND RATIONALE

Intraarticular injection of chymopapain into the rabbit knee joint results in cartilage degradation with rapid loss of proteoglycans. A transient inflammation shortly after injection normally subsides after 1–2 days. Severity and reversibility of the cartilage damage can be altered using different protocols (Williams et al. 1992).

PROCEDURE

Most authors use New Zealand White rabbits at the age of 2 to 4 months. Male Chinchilla and Chinchilla Bastard rabbits are preferred, as they display a thicker cartilage than the New Zealand strain. The animals with an initial body weight of ca 2.5 kg are anesthetized with a continuous inhalation of 4% halothane – 3 l/min N_2O – 1.8 l/min O_2. Both knee joints are carefully shaved and moisturized with 70% alcohol. With a sterile syringe a volume of 0.1 ml of the following solution is injected laterally into the joint space: eighteen mg chymopapain (Sigma) are dissolved in 1 ml 0.9% NaCl, and 50 mg l-cysteine-HCl is added for activation. The preparation is then passed under sterile conditions through a 0.22 µm Millipore filter, and tested for activity prior to each operation. (Potential direct interference of the therapeutic agent to be tested with the chymopapain can also be addressed at that point). One injection of 1.8 mg chymopapain in 0.1 ml per joint usually results in a proteoglycan loss/cartilage dry weight of ca 40% after 10 to 12 days. Two to 4 animals per experiment receive chymopapain in 0.9% NaCl in one joint, and 0.9% NaCl into the contralateral knee to assess the proteoglycan loss caused by the enzyme. Care is taken with all injections to apply the same volume to both joints, as the contralateral serves as internal control. If the drug is given orally, only one knee receives chymopapain treatment, whereas if the drug is applied intraarticularly into one knee (1 to 5 injections of 0.1 ml volume each, vehicle into contralateral joint, in a period of 5 to 10 days), both knees are treated with chymopapain. The animals are sacrificed after 10 to 14 days, and from defined regions of weight-bearing areas in the joint full thickness samples are obtained for histology and assessment of proteoglycan content.

Histology is performed on full thickness sections of the articular cartilage, fixed with 3% formalin, embedded in paraffin, and stained routinely with safranin-O/fast green, or with toluidin-blue (Romeis 1989).

The proteoglycan content is determined with the dimethyl-methylen blue (DMB) method modified after Farndale et al. (1986) and Chandrasekhar et al. (1987). The wet weight of the cartilage samples is determined immediately after preparation, and their dry weight is recorded after 3 day drying at 60 °C. Samples are then soaked overnight in 1 ml buffer (containing 20 mM disodium-hydrogen-phosphate, 1 mM EDTA, and 2 mM dithiothreitol in 500 ml aqua bidest at pH 6.8). Ten µl of papain, suspended in 0.05 M sodium-acetate (Sigma 9 001-73-4), are then added, and incubated at 60 °C for 6 h. Ten µl of standard (shark chondroitinsulfate C (Sigma C4 384) in a concentration range from 10–200 µg/ml buffer) and of each probe are then transferred into a 96 well microtiter-plate. Two hundred µl of DMB is added, and, after 1 min, the extinction recorded photometrically at 690/540 nm. The DMB solution is prepared by dissolving 16 mg DMB (Serva 20 335) in 2 ml methanol, adding 400 ml aqua bidest, 9.6 ml 1N HCl-solution, 3.04 g glycine, and 2.36 g NaCl, stirred and warmed until dissolved, and then diluted to a final volume of 1 000 ml with aqua dest (pH 3.0). The results are recorded as µg chondroitin-sulfate equivalents/mg cartilage dry weight.

EVALUATION

The histological appearance of the articular cartilage is assessed with a modified Mankin score, emphasizing more earlier changes, and separating degradative aspects, (e.g. loss of safranin staining), from repair aspects, (e.g. the occurrence of pericellular staining). The proteoglycan content is expressed as % change to the contralateral knee, or, comparing groups, to chymopapain-treated or. untreated control groups. To relate the proteoglycan content to cartilage dry weight has been found to be more reliable than choosing wet weight as baseline, as the cartilage water content is known to change in this model according to matrix composition.

CRITICAL ASSESSMENT OF THE TEST

In this model, hyaluronic acid preparations and some MMP inhibitors can be detected, NSAIDs do not change the parameters tested. The protocol can be modified to emphasize repair phenomena, or to assure a more severe course of cartilage loss (Williams et al. 1993).

MODIFICATIONS OF THE MODEL

To avoid interaction of a drug with the enzymatic nature of the chymopapain, other degradation mediators can be applied instead, as e.g. fibronectin fragments (Williams et al. 1988).

To ensure continuous synovial levels of a given drug, an osmotic minipump containing the drug can be implanted into the femoral muscles with a prolonged fine tube to insert into the patellar groove (Karbowski et al. in prep). This is an elaborate procedure in which a

Ketavet/Rampun anesthesia is needed, and postoperative plastic collars to prevent the rabbits to interfere with the operated region for the first three days. This results in highly consistent data compared to repeated injections.

Osteoarthritic lesions in the knee joints of male C57bl10 mice were induced by a single intra-articular injection of bacterial collagenase (Van der Kraan et al. 1990; Van den Berg et al. 1993).

Van Osch et al. (1995) developed a device to measure laxicity of knee joints in mice. Reproducible, non-linear S-shaped load displacement curves were determined from knee joints of normal mice. Parameters of anterior-posterior translation, varus-valgus rotation, and compliance were calculated from the curves. Laxicity was marked increased in animals with osteoarthritis induced by intra-articular injection of collagenase.

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I.1.2.4 Spontaneous OA model in STR/1N mice

PURPOSE AND RATIONALE

This spontaneous model has the advantage of a gradually developing OA starting with cartilage surface erosion and fibrillation to osteophyte formation, subchondral bone remodeling, and finally eburnation, in a moderate time frame. All these steps, with only intermittent inflammatory flares, are similar to human pathology, and develop without any interference. The disadvantages are, however, the restriction to systemic drug application, due to the relative small animal size, and the variance in onset and severity of the disease among the animals.

PROCEDURE

The STR/1N mouse strain, characterized extensively by Walton (1977–1979) can be obtained from the National Institute of Health, Bethesda MA, USA. It is not identical to the STR/ORT strain as described by Benjamin et al. (1995), since it displays, in contrast to the ORT, no calcification in ligaments or tendons. Male mice develop OA earlier and more consistently than females, and, without dietary restriction, develop additionally obesity.

Beginning at the age of 10 weeks, male mice are trained to walk on a slowly rotating cylinder (or treadmill, manufactured by Ugo Basili, Italy) recording the mean walking time of each mouse. Mice with a moderate activity (neither dropping off too soon, nor staying on for hours) are then selected to enter the experiment. In groups of 8 to 10 animals, the drug is applied systemically for 8 weeks, and the mobility of each animal is recorded once or twice a week on the rotating cylinder. The body weight is recorded regularly as well. At the end of the experiment, the animals are sacrificed, both knee joints dissected, fixed, decalcified, and embedded in defined orientation for histology.

EVALUATION

The mean walking time decreases with age and disease progression, and is recorded in a time-dependent graph over the treatment period. The medial tibial plateau of the knee joints exhibits the most pronounced cartilage fibrillation and loss, and is, therefore, selected for histological evaluation, based on a modified Mankin grade.

CRITICAL ASSESSMENT OF THE TEST

Since this model is best suitable for oral drug application, experience with disease-modifying drugs for osteoarthritis is limited. Drugs with anti-inflammatory properties increase walking time, but do not alter the

morphological aspects of cartilage degradation in the joint. Analgesic drugs have no effect on either.

MODIFICATIONS OF THE TEST

Several authors (Nakamura 1990; Pataki, Witzemann 1990; Wilhelmi, Meyer 1983) have used the C57black mouse which, however, is reported to vary considerably in the incidence and severity of the disease, and also to develop the osteoarthritis only at older ages.

The STR/ORT strain differs in its calcification of fibrous cartilage (Benjamin et al. 1995), and seems to change the orientation of the proteoglycans in the hyaline cartilage (Dunham et al. 1989).

Brewster et al. (1998) tested an orally active collagenase inhibitor in STR/ORT mice. Microfocal X-ray-generated images of the hind limbs as well as histologic sections of the knees were scored for degradative changes and drug effects.

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I.2 Methods for testing immunological factors[3]

I.2.1 *In vitro* methods

I.2.1.1 Inhibition of histamine release from mast cells

PURPOSE AND RATIONALE

Hypersensitivity reactions can be elicited by various factors: either immunologically induced, i.e. allergic reactions to natural or synthetic compounds mediated by IgE, or non-immunologically induced, i.e. activation of mediator release from cells through direct contact, without the induction of, or the mediation through immune responses. Mediators responsible for hypersensitivity reactions are released from mast cells. An important preformed mediator of allergic reactions found in these cells is histamine. Specific allergens or the calcium ionophore 48/80 induce release of histamine from mast cells. The histamine concentration can be determined with the *o*-phthalaldehyde reaction.

PROCEDURE

Preparation of mast cell suspension

Wistar rats are decapitated and exsanguinated. Fifty ml of Hank's balanced salt solution (HBSS) are injected into the peritoneal cavity and following massage of the body, the abdominal wall is opened. The fluid containing peritoneal cells is collected in a centrifuge tube and centrifuged at 2 000 rpm. The cells are resuspended in HBSS. Then the cell suspension is brought to a final concentration of 10^5 mast cells/100 µl.

Test compound administration and induction of histamine release

1 ml test drug (concentration range between 10^{-4} and 10^{-8} Mol) is added to the mast cell suspension (10^5 cells/100 ml) and the mixture is incubated at 37 °C for 15 min. The cells are made up to a volume of 3 ml with HBSS,

[3] With contributions in the first edition by R. R. Bartlett.

an equal volume of calcium-ionophore (10^{-6} g/ml), compound 48/80 or specific allergen is added. The suspension is incubated at 37 °C for 30 min followed by centrifugation at 2 500 rpm.

The following control solutions are needed

- *Spontaneous histamine release:* contains only mast cells and solutions used to determine baseline.
- *Histamine release:* contains mast cells and solutions and calcium-ionophore (10^{-6} g/ml).
- *Test compound control:* contains solutions and test compound to test the compound for native fluorescence.
- *Solution control:* contains only solutions used in the test to determine baseline.

Extraction of histamine

One ml of the top layer is transferred to a tube containing 300 mg NaCl and 1.25 ml butanol. The sample is alkalized to extract the histamine into butanol by adding 1 ml 3 N NaOH. Following mechanical shaking, the sample is centrifuged for 5 min. One ml of the top layer (butanol) is pipetted into a 5 ml tube containing 2 ml of n-heptane and 0.4 ml of 0.12 N HCl. The tube is mixed by inverting it several times. Following separation into aqueous and organic phases, 0.5 ml of the aqueous phase is transferred to another tube.

Induction of o-phthaladehyde complexing reaction

To each sample 100 µl 1 N NaOH is added under constant stirring immediately followed by administration of 100 µl 0.2% *o*-phthalaldehyde solution. After 2 min, the *o*-phthalaldehyde complexing reaction is stopped by addition of 50 µl 3 N HCl.

Determination of histamine release

The total sample is transferred to an autosampler vial and the histamine concentration is determined by a fluorescence detector (using excitation and emission wave lengths of 350 and 450 nm respectively).

EVALUATION

Percent histamine release (hist. rel.) can be expressed by the following formula:

$$\frac{\text{sample hist. rel.} - \text{spontaneous hist. rel.}}{100\%\ \text{hist. rel.} - \text{spontaneous hist. rel.}} \times 100$$

The statistical evaluation is carried out using the Student's *t*-test (comparison of 100% control to experimental group)

CRITICAL ASSESSMENT OF THE METHOD

Disodium cromoglycate has been reported to inhibit the release of histamine and the degranulation of rat mast cells (Orr and Cox 1969; Orr et al. 1971; Johnson and Bach 1975; Church and Young 1983). However, this effect of disodium cromoglycate and its analogues does not parallel the clinical efficacy (Kay et al. 1987).

MODIFICATIONS OF THE METHOD

Johnston et al. (1978) studied the increased superoxide anion production by immunologically activated and chemically elicited macrophages.

Flint et al. (1985) found a significant inhibition of histamine release by disodium cromoglycate in human mast cells recovered by bronchoalveolar lavage.

Ali et al. (1985) investigated the histamine release from rat peritoneal mast cells, human basophil and neutrophil leukocytes, mast cells from mesentery lung and heart of rats and guinea pigs by the skin irritating constituents thapsigargin and thapsigargicin from the resin of the umbelliferous plant *Thapsia gargania.*

Eady (1986) studied the reactivity of mast cells in bronchoalveolar lavage fluid of macaques repeatedly infected with *Ascaris suum*.

Wells et al. (1986) compared release of histamine, LTC_4, and PGD_2 from primate bronchoalveolar mast cells with that of rat peritoneal mast cells.

The release of β-hexosaminidase from mouse or rat bone marrow derived mast cells and from rat peritoneal mast cells was studied by Broide et al. (1986).

Peretti et al. (1990) recommended flow cytometry to investigate mast cell degranulation. Peptides, including substance P and bradykinin analogs release histamine from human skin mast cells (Lawrence et al. 1989).

Williams et al. (1991) studied the vancomycin-induced release of histamine from rat peritoneal mast cells and a rat basophil cell line (RBL-1).

A sensitive colorimetric assay for the release of tryptase from human lung mast cells *in vitro* has been described by Lavens et al. (1993).

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I.2.1.2 Mitogen induced lymphocyte proliferation

PURPOSE AND RATIONALE

Cultured lymphocytes can be stimulated to a proliferative response and to DNA synthesis by various mitogens. Measurement of DNA synthesis can be accomplished by pulse-labeling the culture with tritiated thymidine (^{3}H-thymidine), a nucleoside which is incorporated into the newly synthesized DNA. Immunmodulating properties can be detected either by pretreatment of the animals *in vivo* or by adding the test drug to the cultured lymphocytes.

PROCEDURE

Mice of NMRI strain weighing 18–20 g or rats of Lewis strain weighing 180–200 g are used.

Materials

Sheep red blood cell (SRBC) specific antigen and/or the following mitogens:

- Lipopolysaccharide 10–0.1 µg/ml
- Dextran sulfate 30–7.5 µg/ml
- Phytohaemagglutinin 0.5–0.12 % stock solution
- Concanavallin A 0.5–0.12 µg/ml
- As standards levamisole, cyclosporine A, prednisolone or leflunomide are used.

Ex vivo

Animals receive the test compound once a day for 5 days. Thereafter, they are sacrificed, spleens are removed and a single cell suspension of 5×10^6 cells/ml is prepared. Mitogens are titrated (4 replicates/group) in 0.1 ml/well and 0.1 ml of the cell suspension is added. Plates are incubated at 37 °C in 5% CO_2 in air for 48–60 h and for another 8 h after addition of 0.25 µC ^{3}H-thymidine per well. Cells are harvested on glass fiber filters and after drying the degree of radioactivity is determined using a β-counter.

In vitro

Animals are sacrificed and their spleens removed. A single cell suspension of 10^7 cells/ ml is prepared and 0.05 ml placed in each microtiter well (4 replicates/ group). Then the test compound (4 times concentrated) is added in 0.05 ml. At last 0.1 ml of the double concentrated mitogen is added. Plates are incubated and processed as described above.

EVALUATION

Stimulation index = proliferation ratio according to positive control, either with or without mean spleen weight. Statistical evaluation is carried out using the Student's *t*-test (comparison of positive and/or negative control to experimental group).

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I.2.1.3 Inhibition of T cell proliferation

PURPOSE AND RATIONALE

Activation and/or proliferation of clonal populations of T cells are critical for the initiation of an antigen-specific immune response. Thus, inhibition of T cell activation provides a potent means for suppressing specific immune response. A number of immunosuppressive agents exhibit the ability to suppress T cell activation.

PROCEDURE

Purification of peripheral blood leukocytes and T cells

Peripheral blood leukocytes from normal donors are separated on Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Leukocyte suspensions are washed in HBSS and are resuspended in RPMI 1664 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin/streptomycin. Leukocyte suspensions are resuspended in RPMI 1664 containing 10% heat-inactivated pooled human serum. Highly enriched T cells are obtained by passing leukocytes through a nylon wool column to remove macrophages and B cells and then depleted of NK and monocytes with anti-Leu 11 b (Becton Dickinson, Mountain View, CA) plus complement (Pel-Freeze, Brown Deer, WI). These highly enriched T cells are approximately 95% CD3+ cells, the remaining cells being B lymphocytes.

Mixed lymphocyte reaction

Peripheral blood leukocytes are incubated at 2×10^5/well with equal numbers of gamma-irradiated (3000 rads) allogenic peripheral blood leukocytes and various concentrations of test compounds. Assays are performed in triplicate in 96-well, U-bottom plates. After 6 days of coculture, the cells are pulsed for 6 h with 1 μC of [^{3}H]thymidine per well. [^{3}H]Thymidine incorporation is then measured by scintillation counting. Data are presented as:

$$\%\ \text{inhibition} = \frac{CPMexpt - CPMbckgrd}{CPMctrl - CPMbckgrd} \times 100$$

where *CPMexpt* are mean counts per min of experimental cultures, *CPMbckgrd* are mean counts per min of background well, unstimulated cultures, and *CPMctrl* are mean counts per min of uninhibited, stimulated cultures.

Lymphocyte stimulation and proliferation

Peripheral blood leukocytes and isolated T cells are cultured with anti-CD3 (5 ng/ml) plus PMA (5 ng/ml), anti-CD28 (1: 5 000 dilution) plus PMA (5 ng/ml), or 100 U/ml rhuIL-2 in RPMI 1644 containing 10% fetal bovine serum. Peripheral blood leukocytes or T cells are cultured at 2×10^5 cell per well in a total volume of 200 μl/well. Assays are performed in quadruplicate in 96-well, U-bottom plates. [^{3}H]Thymidine (1 μC) is added to each well after 48 h of coculture and after a 20 h pulse of [^{3}H]thymidine, the cells are harvested and the amount of [^{3}H]thymidine uptake is quantitated on a scintillation counter.

ELISA assays

Supernatants/well (100 mml) are harvested 24 h after initiation of cultures of peripheral blood leukocytes or T cells stimulated with anti-CD3 or anti-CD28 plus PMA. IL-2 in the coculture supernatant is quantitated using a commercially available IL-2 ELISA kit. All experiments are performed in duplicate.

IL-2R assays

The expression of IL-2R on T cells stimulated for 48 h with anti-CD3 or anti-CD28 plus PMA is determined using FITC-conjugated anti-CD25 mABs (Becton Dickinson, Mountain View, CA). T cells are washed in HBSS and then stained with phycoerythrin-conjugated anti-CD3 mAB and fluorescein-conjugated antiCD25 mAB. The percent of cells coexpressing CD3+ and CD25+ are determined from 2000 cells using an EPICS C flow cytometer (Coulter, Hialeah, FL).

EVALUATION

Dose response curves of inhibition of one-way mixed lymphocyte reaction and of IL-2 in the supernatant after stimulation with antiCD3 or anti-CD28 are established.

MODIFICATIONS OF THE METHOD

Zielinski et al. (1993, 1994) studied the influence of leflunomide on expression of lymphocyte activation ex-

pression markers (IL-2 and transferrin receptors) as well as on cell cycle and on IL-2 receptor gene expression.

Calcineurin was found to be a key signaling enzyme in T lymphocyte activation and the target of immunosuppressive drugs (Clipstone and Crabtree 1993).

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I.2.1.4 Chemiluminescence in macrophages

PURPOSE AND RATIONALE

The stimulation of macrophages by antigen, complement, phorbol-esters, ect. leads to elaboration of O_2^- and other oxygen metabolites. Superoxide ion (O_2^-) and other highly reactive oxygen metabolites (radicals) form the basis for an efficient microbicidal system *in vivo*. Yet, when these radicals are released in response to self-antigens, tissue damage is often the result. Inhibition of this process can be regarded as a measure for immunmodulating effects of compounds. The oxygen metabolites can produce light-emitting reactions (chemiluminescence), which is measurable if amplified with suitable agents, such as the cyclic hydrazide luminol.

PROCEDURE

NMRI mice weighing 30 g or Sprague-Dawley rats weighing 250–300 g of either sex are used.

Positive control

1. Sensitized mice, receiving vehicle
2. Mice, developing an autoimmune disease, receiving vehicle
3. Rats, developing adjuvant arthritis, receiving vehicle

Negative control

1. Mice not sensitized, receiving vehicle
2. Mice, not developing an autoimmune disease, receiving vehicle
3. Rats without adjuvant arthritis.

Materials

- 5×10^8 SRBC (sheep red blood cells)/0.5 ml 0.9% NaCl solution (for sensitization)
- *Phorbolester:* Stock solution of 1 mg/ml phorbolmyristenacetate. This stock solution is diluted with Hank's balanced salt solution to a final concentration of 3.5 µM (working solution). For the induction of chemiluminescence, the working solution is diluted in the test tube 1 : 4, resulting in a final phorbolester concentration of 0.875 µM.
- Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma) final concentration 25 µg /ml

Ex vivo experiment

Groups of 6 animals are treated for 6 days orally or subcutaneously with test compound or the standard (prednisolone acetate or leflunomide). They are decapitated and exsanguinated. Macrophages are obtained by flushing the peritoneal cavity with 10 ml saline, containing 250 IU heparin. The cells are pooled, washed several times and suspended again at a final concentration of 2×10^6/200 µl.

For measurement in the luminometer the following mixture is prepared:

200 µl macrophages (2×10^6)
100 µl luminol solution (100 µg/ml)
100 µl phorbolmyristenacetate solution (3.5 µM)

Each sample is mixed thoroughly without the phorbolmyristenacetate solution, put into the luminometer and counted at 2 min intervals for 10 s. The addition of the phorbolester induces the reaction.

In vitro experiment

To 100 µl of macrophage suspension (2×10^6 cells) are 100 µl of the solution of the test compound added and incubated for 15 min at 37 °C.

Then, 100 µl of luminol solution (100 µg/ml) and 100 µl of the 3.5 µM phorbolester solution are added and the luminescence measured in the luminometer.

EVALUATION

The time of maximal counts for the positive control is recorded. For all groups the ratio of counts per 10 s is determined at that time, compared to the positive control counts per 10 s and the percent change is calculated. For statistical evaluation the experimental group is compared with the positive control group using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Bird and Giroud (1985) described a technique of polymorphonuclear leukocyte chemiluminescence as a means to detect compounds with anti-inflammatory activity. Inflammatory polymorphonuclear leukocytes were obtained by injecting rats intrapleurally with 1 ml of a 1% solution of calcium pyrophosphate and collection of the pleural exudate 4 h later. Chemoluminescence responses were measured using a Packard Picolite chemoluminometer and opsonized zymosan as the stimulus.

Seeds et al. (1985) found an independent stimulation of membrane potential changes and the oxidative metabolic burst in polymorphonuclear leukocytes.

A microtechnique for studying chemiluminescence response of phagocytes using whole blood was described by Selvaraj et al. (1982).

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I.2.1.5 PFC (plaque forming colony) test *in vitro*

PURPOSE AND RATIONALE

Identification of antibody producing cells is based on the ability of the secreted IgM antibody to fix complement and thereby lyse the indicator erythrocytes. Spleen cells or peripheral blood lymphocytes, previously incubated with antigen, are mixed with sheep red blood cells (SRBC). After addition of compliment and incubation, plaques (clear areas) caused by the lysis of SRBC appear in the otherwise cloudy layer. Antibody forming cells can be detected by the appearance of plaques. The number of plaques obtained is proportional to the number of antibody producing lymphocytes in the cell population.

PROCEDURE

NMRI mice weighing 16–18 g or Lewis rats weighing 180–200 g of either sex are used.

Materials

- absorbed guinea pig complement
- SRBC stored in Alsever's solution

Positive control

Spleen cells incubated with antigen and medium

Negative control

Spleen cells incubated with medium alone. The animals are decapitated and the spleens are removed from the peritoneal cavity. A single cell suspension of 15×10^6 cells/ml is prepared. For the induction of PFC, a 0.5 ml splenocyte suspension is added to 0.5 ml of a suspension of SRBC, previously washed in medium and diluted to 8×10^6 cells/ml. Thereafter, 1 ml of the solution of the test compound is added and the limbrowells are incubated at 37 °C in a CO_2 incubator for 5 days. Per group 3 limbrowells are set up. On day 5, the 3 wells of each group are pooled, washed in medium and the number of cells is determined. For each cell pellet, 875 µl of washed SRBC and 125 µl absorbed guinea pig compliment are added. The suspension is mixed thoroughly and filled in chambers constructed of microslides. The chambers are placed in the incubator at 37 °C for 90–120 min. The plaque forming colonies are counted immediately after incubation.

EVALUATION

The activity of test compounds can be determined using the following formula:

1. PFC/3 wells:

$$x = \frac{\text{plaques} \times 100}{\mu\text{l}}$$

2. % change in the number of plaques:

$$x = \frac{\text{plaques} \times 100}{\text{plaques pos. control}}$$

$$d\% = x - 100$$

3. % change in number of cells:

$$x = \frac{\text{number of cells} \times 100}{\text{number of cells pos. control}}$$

$$d\% = x - 100$$

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I.2.1.6 Inhibition of dihydro-orotate dehydrogenase

PURPOSE AND RATIONALE

Dihydro-orotate dehydrogenase catalyzes the fourth committed step in the de novo biosynthesis of pyrimidines. As rapidly proliferating human T cells have an exceptional requirement for de novo pyrimidine biosynthesis, small molecule dihydro-orotate dehydrogenase inhibitors constitute an attractive therapeutic approach to autoimmune diseases, immunosuppression, and cancer. The main mode of action of the immuno-suppressive compound leflunomide and its active metabolites is considered to be the inhibition of the enzyme dihydro-orotate dihydrogenase (Bruneau et al. 1998; Graul and Castañer 1998; Knecht and Löffler 1998; Rückemann et al. 1998; Schorlemmer et al. 1998; Herrmann et al. 2000; Liu et al. 2000).

PROCEDURE

A fragment of human dihydro-orotate dehydrogenase is expressed by means of the baculovirus expression vector system and purified to a specific activity greater than 50 U/mg (Knecht et al. 1996, 1997). Enzyme assays are performed with purified recombinant dihydro-orotate dehydrogenase at 30 °C. The oxidation of the substrate dihydro-orotate and the reduction of the co-substrate quinone is coupled to the reduction of the chromogen 2,6-dichlorophenolindophenol (DCIP). The reaction mixture contains 0.1 mM Q_D or 0.1 M Q_{10}, 1 mM L-dihydro-orotate, 0.06 mM DCIP, 0.1% Triton X-100 in 50 mM Tris-HCl buffer, 150 mM KCl, pH 8.0. The reaction is started by addition of the enzyme. The loss of absorbance of the blue DCIP is monitored at 600 nm; $\epsilon = 18.800\ \text{l mol}^{-1}\,\text{cm}^{-1}$. The enzyme activity in control assays without Q_D or Q_{10} which is approximately 1% of maximum enzyme activity is subtracted from the activity values measured. Stock solutions of the test compounds are prepared in dimethylsulfoxide with further dilutions in the buffer taken for the assays.

EVALUATION

To determine the inhibitory potency of the agents, the initial velocity of dihydro-orotate dehydrogenase reaction is measured at saturating substrate concentrations, 1 mM dihydro-orotate and 100 µM Q_D and varying concentrations of the drugs (1 nM through 100 µM). The equation is fitted to the initial velocities:

$$v = V / \{1 + [\text{I}]/IC_{50}\}$$

([I] is the inhibitor concentration) in order to find the concentration causing 50% inhibition of the enzyme activity (IC_{50}).

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I.2.2
In vivo methods for testing immunological factors

I.2.2.1
Spontaneous autoimmune diseases in animals

Several spontaneous autoimmune diseases have been reported in several inbred animal strains:

New Zealand black mouse (NZB mouse) (Bielschowski et al. 1959; Howie and Helyer 1968; Barthold et al. 1974; Blanchard and Bach 1980). The NZB mouse develops a spontaneous autoimmune disease with autoimmune hemolytic anemia, splenomegaly, glomerulonephritis, lymphoproliferative disorders and peptic ulcerations.

New Zealand black/white F1 (B/W) mouse (Helyer and Howie 1963; Kessler 1968): These animals develop nephritis similar to that in human systemic lupus erythematosus and show mononuclear cell infiltration in salivary and lachrymal glands such as in human Sjögren's syndrome.

A substrain of the autoimmune-prone mouse, NZB/kl, was found to show spontaneous elevation of the auditory brainstem response threshold with age (Sone et al. 1995).

Immunodeficient alymphoplasia mice were recommended as a spontaneous model for Sjøgren's syndrome (Tsubata et al. 1996). Mice homozygous for an autosomal recessive mutation aly (alymphoplasia) lack both lymph nodes an Peyer's patches and show defects in both humoral and cellular immunity. Histo-pathological analyses revealed chronic inflammatory changes in exocrine organs such as the salivary gland, lacrimal gland and the pancreas.

The **Palmerston North autoimmune mouse strain** which exhibits both spontaneous systemic autoimmune disease and otic capsule bone formation has been proposed as a model for otic capsule osteogenesis and otosclerosis (Hertler and Trune 1990; Traynor et al. 1992).

In aging **BDF1 mice**, Hayashi et al. (1988) described spontaneous development of autoimmune sialadenitis.

Robison et al. (1994) examined the relationship between orchitits and aspermatogenesis in various strains of H_2 congenic mice and defined a genetic predisposition to spontaneous aspermatogenesis.

Motheaten mice. Mice homozygous for the autosomal recessive motheaten (me) or the allelic viable motheaten (me^v) mutations develop severe and early-age onset of systemic autoimmune and inflammatory disease (Green and Shultz 1975; Shultz et al. 1984; Shultz 1988; Su et al. 1998).

The genetic, hormonal and behavioural influence on spontaneously developing arthritis in normal mice has been reviewed by Holmdahl et al. (1992).

Non-obese diabetic mouse (NOD mouse) (Makino et al. 1980; Miyazaki et al. 1985; Leiter et al. 1987). The inbred NOD mouse is considered a good model for type I diabetes mellitus. Mononuclear cells infiltrate the pancreatic islets of Langerhans from 6–8 weeks of age, followed by a progressive and selective destruction of insulin-producing β-cells and the onset of IDDM from the 12^{th} week of age onwards.

Itoh et al. (1997) studied the requirement of Fas for the development of autoimmune diabetes in nonobese diabetic mice.

Quartey-Papafio et al. (1995) showed that aspartate at position 57 of nonobese diabetic I-A(g7) β-chain diminishes the spontaneous incidence of insulin-dependent diabetes mellitus in the NOD mouse.

The NOD mouse was also recommended to study the pathogenesis of autoimmune thyroiditis (Many et al. 1996).

Bio-breeding rat (BB rat) (Like et al. 1982; Field 1983; Yale and Marliss 1984). On the basis of clinical and histopathological parameters, the BB rat is considered a useful model for human IDDM. The disease in the BB Rat is characterized by infiltration of lymphocytes and macrophages into the islets of Langerhans.

Allen and Thupari (1995) described spontaneous autoimmune lymphocytic thyroiditis in *BB/Wor rats.*

Obese strain chicken (OS chicken) (van Tienhoven and Cole 1962; Cole 1966; Cole et al. 1968, 1970; Wick et al. 1974). The OS chicken is perhaps the best studied model for an organ-specific, spontaneously occurring autoimmune disease, viz. spontaneous autoimmune thyroiditis, which closely resembles human Hashimoto thyroiditis. The spontaneous autoimmune thyroiditis in obese chicken was further studied by Neu et al. 1986; Kroemer et al. 1989; Cihak et al. 1995; Hala et al. 1996; Dietrich et al. 1997.

Chickens of the University of California line 200 **(UCD-200 chickens)** develop an inherited inflammatory fibrotic disease that closely resembles human progressive systemic sclerosis (scleroderma) (Gershwin et al. 1981; Van de Water et al. 1984; Brezinscheck et al. 1993).

Schumm-Draeger and Fortmeyer (1996) described **autoimmune thyroiditis in the cat** as a spontaneous disease model.

Spontaneous autoimmune thyroiditis was found in **Mastomys** (*Praeomys coucha*) by Solleveld et al. (1985) and recommended as an animal model of human disease.

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I.2.2.2 Acute systemic anaphylaxis in rats

PURPOSE AND RATIONALE

Rats are immunized with ovalbumin and *Bordetella pertussis* suspension as adjuvant. After 11 days the animals are challenged by intravenous injection of ovalbumin. The shock symptoms can by inhibited by corticosteroids and intravenous disodium cromoglycate.

PROCEDURE

Female Sprague-Dawley rats weighing 120 g are immunized by i.m. injection of 10 mg/kg highly purified ovalbumin. Simultaneously 1 ml of *Bordetella pertussis* suspension (2×10^{10} organisms) is injected intraperitoneally. IgE antibodies are induced and attached to the surface of mast cells and basophilic granulocytes. Eleven days later the animals are challenged by intravenous injection of 25 mg/kg highly purified ovalbumin. This results in formation of antigen-antibody-complexes on the surface of mast cells and basophilic granulocytes in blood and in all organs with immediate release of various mediators of anaphylaxis, such as histamine, serotonin, SRS-A, prostaglandins; in shock symptoms and 80% lethality. Corticosteroids, e.g. dexamethasone 1–10 mg/kg s.c. are given 18 h prior to challenge, or 30 mg/kg disodium cromoglycate i.v. before injection of ovalbumin. Ten–20 animals are used for each group.

EVALUATION

The shock symptoms are scored and mortality counted. Results after treatment are compared with untreated controls. Pretreatment with corticosteroids or disodium cromoglycate can inhibit death and ameliorate shock symptoms. Statistical calculation is performed using the χ^2-test.

MODIFICATIONS OF THE METHOD

Desensitization by repeated 'microshocks' of constant strength in guinea pigs has been reported by Herxheimer (1952).

Acute systemic anaphylaxis experiments have also been performed in guinea pigs and in mice. In guinea pigs anaphylactic bronchospasm can be measured with the Konzett and Rössler method (see D.2.2.1) (Davies and Evans 1973).

Moreover, anaphylactic bronchospasm can be measured in isolated guinea pig lungs according to the method of Bhattacharya and Delaunois (1955).

Anaphylaxis can be measured in the chopped guinea pig lung by assay of the supernatant in the isolated guinea pig ileum in the presence of 2×10^{-7} M atropine (Austen and Brocklehurst (1961).

Ufkes and Ottenhof (1984) sensitized Brown-Norway rats with a suspension of trinitrophenyl haptenized ovalbumin together with $AlPO_4$ as adjuvant. Bronchial and cardiovascular function were studied after treatment with anti-allergic agents and antigen challenge.

Elwood et al. (1992) studied the effect of dexamethasone and cyclosporin A on allergen-induced airway hyperresponsiveness and inflammatory cell responses in sensitized Brown-Norway rats.

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I.2.2.3 Anti-anaphylactic activity (Schultz-Dale reaction)

PURPOSE AND RATIONALE

Guinea pigs are sensitized against egg albumin. Challenge after 3 weeks causes in isolated organs release of mediators, e.g. histamine, which induce contraction in isolated ileum.

PROCEDURE

Guinea pigs of either sex weighing 300–350 g are sensitized with alum precipitated egg albumin. Alum egg albumin is prepared by dissolving egg albumin (1 mg/ml) in six percent aluminum hydroxide gel, suspended in saline. The mixture is stirred and kept at room temperature. Each animal receives at the same time injections of 0.125 ml of this mixture in each foot pad and 0.5 ml subcutaneously. After 4 weeks the animals are killed and the ileum is dissected out. Cleaned pieces, about 2–3 cm long, are mounted in an organ bath containing Tyrode solution at 37 °C. The strips are allowed to equilibrate for 15 min. The contractility of the ileum strips is tested by adding 10^{-4} g/ml $BaCl_2$ solution. To one organ bath the standard (2×10^{-6} g/ml final concentration of Tribenosid = 1-O-ethyl-3,5,6-tri-O-benzyl-D-glucofuranoside = Glyvenol® CIBA) and to other vials the test compounds (final concentration up to 10^{-5} g/ml) are added. One organ bath serves as control. After 3 min ovalbumin in a final concentration of 2×10^{-6} g/ml is added. The contractions are recorded with strain gauges by a polygraph.

EVALUATION

The results are expressed as presence or absence of blocking activity (percentage inhibition). If anti-anaphylactic activity is observed, ED_{50} values using different doses are calculated.

CHRITICAL ASSESSMENT OF THE METHOD

Positive results can also be achieved with spasmolytics, local anesthetics, antihistaminics, and sympathicomimetics.

MODIFICATIONS OF THE METHOD

The method has been modified by testing histamine release in the lung after challenging with egg albumin. Either lung strips from sensitized guinea pigs are suspended in an organ bath and their contractions are measured after addition of egg albumin or the entire lung tissue is dissected out and washed free from blood by perfusing with warm oxygenated Tyrode solution via the pulmonary artery. The lung tissue is chopped and washed with Tyrode solution in order to remove the remaining blood. The chopped lung tissue is divided into 24 samples, each of approximately 100 mg wet weight. These are incubated at 37 °C in Tyrode solution for 15 min with continuous agitation by rocking, after which, 1 mg/ml of egg albumin is added to the reaction mixture. After shaking for 10 min at 37 °C, the supernatant is collected and assayed for histamine with guinea pig ileum. Atropine sulfate 2 mg/ml is added in Tyrode solution. The residual histamine is obtained by boiling the tissue in 5 ml Tyrode solution for 10 min. The tubes are then placed on ice for 1 h to allow complete diffusion. Released histamine is expressed as a percentage of total histamine content.

Koppel et al. (1981) developed a method to induce contraction of immunologically sensitized mouse trachea by antigen (Schultz-Dale reaction).

The trachea of sensitized guinea pigs was used by Omote et al. (1994).

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I.2.2.4 Passive cutaneous anaphylaxis

PURPOSE AND RATIONALE

Passive cutaneous anaphylaxis is a immune reaction of the immediate type. By passive immunization of rats in the skin with rat anti-ovalbumin serum and a challenge 2 days later with ovalbumin at the same skin area antigen-antibody complexes are formed in the mast cells inducing release of mediators. This results in vasodilatation, increase in permeability of the vessel walls and leakage of plasma. To make the allergic reaction visible, Evan's blue dye is administered along with the antigen. Evan's blue dye is attached to the albumin fraction of plasma, producing a blue spot. This blue spot indicates that an anaphylactic reaction has taken place in the skin.

PROCEDURE

For preparation of antiserum male rats weighing 200–250 g are adrenalectomized and are allowed to recover for 3 days. Thereafter, animals are sensitized with egg albumin (1 mg/animal) using aluminium hydroxide gel (200 mg) as adjuvant. Alum egg albumin is prepared by dissolving 1 mg/ml of egg albumin in 20% aluminium hydroxide gel, suspended in saline. Each animal simultaneously receives 0.125 ml of the above solution in each foot pad and 0.5 ml subcutaneously. After 8 days, the animals are bled and antiserum is collected.

For the test, the antiserum is diluted in such a manner as to give a wheal of 15–20 mm diameter in a preliminary titration. Aliquots of 100 µl of appropriate dilution of antiserum are injected intradermally into the shaved dorsal skin of normal male rats weighing about 100 g. After 24 h of latent period each animal is challenged with the intravenous administration of 0.1 ml of 2.5% Evans blue dye containing 25 mg/ml of egg albumin. In the case of intravenous administration, the test compound is administered simultaneously with the antigen and the dye. In case of oral testing, the compound is given orally 1 h prior to challenge. The animals are sacrificed 30 min after the challenge. The amount of Evans blue dye leaked at the site of passive cutaneous anaphylactic reaction is extracted and determined colorimetrically at 620 mµ wavelength.

EVALUATION

The amount of Evans blue extracted from passive cutaneous anaphylactic reaction is taken as 100 percent. Percent inhibition of passive cutaneous anaphylactic reaction in the rats treated with the test compound is calculated. The standard disodium cromoglycate at a dose of 3 mg/kg i.v. or 30 mg/kg orally results in 80–100% inhibition. Using different doses, ED_{50} values can be calculated.

MODIFICATIONS OF THE METHOD

Goose and Blair (1969) used *Bordetella pertussis* and extracts of the worm *Nippostrongylus brasiliensis* as antigens in passive cutaneous anaphylaxis experiments in the rat.

Patterson et al. (1971) tested passive cutaneous reactivity to antihuman IgE in rhesus monkeys.

Without immunization, plasma extravasation after bradykinin injection can be tested in anesthetized Sprague-Dawley rats (Lembeck et al. 1991). Evans blue dye is injected to stain plasma proteins. After injection of bradykinin antagonists followed by bradykinin injection, the rats are perfused with physiological saline. The trachea, the urinary bladder, and the duodenum are resected, weighed and incubated for 48 h in formamide at 50 °C (Saria et al. 1983). The amount of Evans blue extracted is measured photometrically at 620 nm.

Vascular reactions to histamine, histamine-liberator and leukotaxine in the skin of guinea pigs using pontamine sky blue 6X as indicator were studied by Miles and Miles (1952).

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I.2.2.5 Arthus type immediate hypersensitivity

PURPOSE AND RATIONALE

The immune complex induced Arthus reaction comprises inflammatory factors that have been implicated in the acute responses in joints of rheumatic patients. Complement and polymorphonuclear neutrophiles are activated via precipitating antigen-antibody complexes leading to an inflammatory focus characterized by edema, hemorrhage and vasculitis. Arthus reaction of the immediate type becomes maximal 2–8 h after challenge.

PROCEDURE

Ovalbumin suspension

1 700 mg ovalbumin are suspended in 100 ml paraffin oil. 4.38 ml pertussis vaccine are suspended in 70 ml 0.9% NaCl-solution. Both suspensions are mixed to form an emulsion.

Wistar or Sprague-Dawley rats of either sex weighing 220–280 g can be used. Seven days prior to start of the experiment rats are sensitized by i.m. administration of 0.5 ml of the ovalbumin suspension. They are housed in groups of eight with standard food and water ad libitum.

Twenty-four hours and one hour prior to induction of the Arthus reaction, test compounds are administered to groups of 8 animals. The rats are challenged by injection of 0.1 ml of 0.04% solution of highly purified ovalbumin in the left hind paw. Swelling of the paw occurs which reaches a maximum after a few hours. The footpad thickness can be measured by calipers. One group of sensitized animals treated with solvent alone serves as positive control, one group of nonsensitized animals treated with solvent alone serves as negative control. Standard doses are 30 mg/kg cortisone or 10 mg/kg prednisolone p.o.

EVALUATION

The change in footpad thickness is expressed as the percent change from the vehicle control group. Comparison of experimental group to positive control is evaluated statistically using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Instead of ovalbumin, sheep red blood cell suspensions can be used for immunization and for challenge in mice (Omote et al. 1994).

Nagakawa et al. (1990) sensitized mice by s.c. injection of bovine serum albumin in complete Freund's adjuvant and boosted on day 21 by an intradermal injection of BSA. On day 28, the Arthus reaction was elicited by intradermal injection of BSA. Four hours later, an erythematous skin reaction over an area of more than 8 mm^2 was regarded as positive.

Kamei et al. (1991) immunized guinea pigs by injection of a mixture of egg albumin and Freund's complete adjuvant subcutaneously into the food pad or i.m. into the hind leg. The injection was repeated 4 times at 7 day intervals. Ten days after the last immunization, 0.2 ml of 2.5% egg albumin was injected sc. into the dorsal skin of the animals. The intensity of the Arthus reaction was evaluated by measuring the inflamed area according to scores.

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I.2.2.6 Delayed type hypersensitivity

PURPOSE AND RATIONALE

Delayed type hypersensitivity is a reaction of cell mediated immunity and becomes visible only after 16–24 h. The same methods as for testing immediate type hypersensitivity can be used.

PROCEDURE

Rats are sensitized in the same way by i.m. administration of 0.5 ml ovalbumin suspension 7 days prior to the start of the experiment as described for testing immediate type hypersensitivity. They are challenged by injection of 0.1 ml of 0.04% solution of highly purified ovalbumin in the left hind paw. Footpad thickness is measured immediately and 24 h after ovalbumin administration.

MODIFICATIONS OF THE METHOD

Mizukoshi et al. (1994) injected female CDF1 mice intradermally with a suspension of 2×10^8 sheep red blood cells/50 µl into the left foot pad. A second booster of the same dose was given to the right foot pad on day 4. The thickness of the foot pads was measured on the following day, and the difference in the thickness between the right and the left food pads was taken as the degree of swelling.

Kamei et al. (1991) immunized mice by applying 0.15 ml of 7% picryl chloride/ethanol solution to the skin of the shaved abdomen. The second immunization was performed 6 days later. One week after the second immunization, 1 drop of 1% picryl chloride olive oil solution was applied to the ear and the thickness of the ear was measured by a thickness gauge 24 h later.

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I.2.2.7 Reversed passive Arthus reaction

PURPOSE AND RATIONALE

In the reversed passive Arthus reaction the antigen is injected intravenously followed by a local injection – either intradermally or into the pleural space – of the respective antibody. Generation of an immune-mediated reverse passive Arthus reaction in the rat pleural cavity results in a classic acute inflammatory response. The methods are used to evaluate new anti-inflammatory agents.

PROCEDURE

Male Lewis rats weighing 200–250 g are fasted overnight prior to use with free access to water. The animals receive 5 mg bovine serum albumin in 0.2 ml sterile saline intravenously, followed 30 min later by injection of 1 mg rabbit anti-BSA in 0.2 ml sterile saline into the right pleural cavity under light halothane anesthesia. Drugs or vehicle controls are administered by gastric gavage in 1 ml/100 g body weight at different times prior to the anti-BSA. The animals are sacrificed at various intervals after anti-BSA injections by CO_2 inhalation (after 5 min for thromboxane B_2 determination, after 10 min for leukotriene B_4 determination, and after 4 h at the peak time of neutrophil infiltration). The fluid exudate is removed from the pleural cavity by gentle vacuum aspiration and the volume is recorded. Eikosanoids in the pleural exudate are quantitated by commercial RIA kits.

EVALUATION

The values after treatment with various doses of test compounds are compared with those of vehicle controls.

MODIFICATIONS OF THE METHOD

The antibody can be injected intradermally into the shaved skin of rats after intravenous injection of the antigen (e.g., human albumin) together with Evans blue dye solution. Extravasated dye is determined in skin punches (Camussi et al. 1990; Burch et al. 1992; Okamoto et al. 1992).

Bailey and Sturm (1983) induced the reverse passive Arthus reaction in rats using bovine serum albumin as antigen into the tail vein and rabbit anti-bovine serum albumin into the skin site. One hour after oral dosing with vehicle or drug, animals were lightly anesthetized and their hair was shaved from the mid-dorsal region with electric clippers. Each animal was injected intradermally with 40 µl on the left side of the mid-dorsal line and with 40 µl of rabbit anti-bovine serum albumin (5.0 mg/ml antibody protein), diluted 1 : 4 with phosphate-buffered saline on the right side of the dorsal midline. Immediately following the intradermal challenge, each rat received 0.5 ml phosphate-buffered saline containing 1.0 mg bovine serum albumin injected in the tail vein. Four hours after intradermal challenge, the animals were sacrificed. The full thickness skin was removed from the back and discs 8 mm in diameter were punched out with a metal punch. Wet weight of the samples from the phosphate-buffered saline- and antibody-injected site was determined and the edema induced by the reverse passive Arthus reaction calculated as the difference between both weights.

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I.2.2.8 Adjuvant arthritis in rats

PURPOSE AND RATIONALE

Adjuvant arthritis in rats has been described by Pearson and Wood (1959) exhibiting many similarities to human rheumatoid arthritis. Injections of complete Freund's adjuvant into the rat paw induces inflammation as primary lesion with a maximum after 3 to 5 days. Secondary lesions occur after a delay of approximately 11 to 12 days which are characterized by inflammation of non-injected sites (hindleg, forepaws, ears, nose and tail), a decrease of weight and immune responses. The procedure has been modified by several authors in order to differentiate between anti-inflammatory and immunosuppressive activity (e.g. Perper et al. 1971). Anti-inflammatory compounds do not inhibit secondary lesions, which are prevented or diminished by immunosuppressive agents. Two protocols, termed "preventative" (or "prophylactic") and "therapeutic" (or "established") adjuvant arthritis, have gained wide usage for assessing a drug's potential anti-arthritic activity (Schorlemmer et al. 1999).

PROCEDURE

The choice of the animal strain has been found to be very important for the performance of this test. Wistar-Lewis rats have been proven to be very suitable in contrast to other sub strains. Male rats with an initial body weight of 130 to 200 g are used. On day 1, they are injected into the sub plantar region of the left hind paw with 0.1 ml of complete Freund's adjuvant. This consists of 6 mg mycobacterium butyricum (Difco) being suspended in heavy paraffin oil (Merck) by thoroughly grinding with mortar and pestle to give a concentration of 6 mg/ml. Dosing with the test compounds or the standard is started on the same day and continued for 12 days. Paw volumes of both sides and body weight are recorded on the day of injection, whereby paw volume is measured plethysmographically with equipment as described in the paw edema tests. On day 5, the volume of the injected paw is measured again, indicating the primary lesion and the influence of therapeutic agents on this phase. The severity of the induced adjuvant disease is followed by measurement of the non-injected paw (secondary lesions) with a plethysmometer. Purposely, from day 13 to 21, the animals are not dosed with the test compound or the standard. On day 21, the body weight is determined again and the severity of the secondary lesions is evaluated visually and graded according the following scheme:

		score
ears:	absence of nodules and redness	0
	presence of nodules and redness	1
nose:	no swelling of connective tissue	0
	intensive swelling of connective tissue	1
tail:	absence of nodules	0
	presence of nodules	1
forepaws:	absence of inflammation	0
	inflammation of at least 1 joint	1
hind paws:	absence of inflammation	0
	slight inflammation	1
	moderate inflammation	2
	marked inflammation	3

EVALUATION

a) For primary lesions: The percent inhibition of paw volume of the injected left paw over vehicle control is measured at day 5.
b) For secondary lesions: The percentage inhibition of paw volume of the non-injected right paw over controls is measured at day 21.
c) An arthritic index is calculated as the sum of the scores as indicated above for each animal. The average of the treated animals is compared with the control group.
d) The total percentage change is calculated as follows by addition of:
percent inhibition of the injected paw on day 5 + percent inhibition of the non-injected paw on day 21 + percent change of the arthritic index.

Doses of 0.3 mg/kg indomethacin p.o. and 20–50 mg/kg phenylbutazone p.o. are effective on the primary lesions when dosage is started at the day of injection of the irritant. They are not effective on the secondary lesions.

In contrast, immunosuppressants like cyclophosphamide at a dose of 7 mg/kg inhibited the secondary lesions even when started at day 9 or later.

CHRITICAL ASSESSMENT OF THE METHOD

Evidence was given that adjuvant arthritis in the rat is associated with chronic pain (Colpaert 1987) The measure of pain in this model still presents some technical problems since the evaluation is based on the somewhat biased observation of the behavioral responses.

MODIFICATIONS OF THE METHOD

A review was given by Gardner (1960) on the experimental production of arthritis.

Moran et al. (1999) compared adjuvant arthritis and selected animal models of arthritis to rheumatoid arthritis with special emphasis on the mechanism of joint destruction.

Kazuna and Kawai (1975) and Rooks et al. (1982) used rats with established lesions to test analgesics in the arthritic flexion pain test. The method is claimed to be specific by detecting only central analgesics and nonsteroidal anti-inflammatory drugs but not other classes such as CNS-depressant or antihistaminic drugs.

Brackertz et al. (1977) established antigen-induced arthritis in the mouse by immunization with methylated bovine serum albumin in complete Freund's adjuvant with B pertussis vaccine.

A streptococcal cell wall-induced arthritis in rats has been described by Wilder et al. (1982, 1987) and Yocum et al. (1986).

Lewis et al. (1997) studied degradation of articular cartilage in a rat monoarthritis model induced by an intra-articular injection of *Propionibacterium acnes*.

Crossley et al. (1989) reported on a monoarticular antigen-induced arthritis in rabbits and mice.

α-2-Glycoprotein levels have been recommended as parameter for severity and inhibition of experimental immunoarthritis in the rat by Sandow et al. (1971).

Pircio et al. (1975) recommended a method for the evaluation of analgesic activity using adjuvant-induced arthritis in rats. The degree of vocalization was recorded from 5 rats placed together in a counting chamber.

Cruwys et al. (1994) sensitized rats on day 0 and 7 with multiple intradermal injections of methylated bovine serum albumin emulsified in Freund's complete adjuvant. On day 21, the animals were challenged by the intra-articular injection of 100 µl 0.5% solution of methylated bovine serum albumin into the right knee. The progress of the monoarticular arthritis was monitored by daily measurement of joint diameter.

Butler et al. (1991) described a limited arthritic pain model for chronic pain and inflammation studies using injections of 0.05 ml of complete Freund adjuvant into the left tibio-tarsal joint of Sprague-Dawley rats.

Esser et al. (1995) measured radiographic changes in adjuvant-induced arthritis in rats by quantitative image analysis. Digitized radiographs of the calcaneus were examined for changes in the mean and in the distribution of gray values. Periostal new bone formation was measured as an increase in image area of the calcaneus.

Mercuric chloride ($HgCl_2$) induces a syndrome of autoimmunity in Brown-Norway rats characterized by a variety of IgG antibodies, very high concentrations of serum IgE, proteinuria, leukocytoclastic vasculitis which predominantly affects the caecum, and an inflammatory polyarthropathy (Kiely et al. 1995, 1996).

Consden et al. (1971), Cook and Jasin (1972), Cook et al. (1972), Jasin and Cook (1977) produced a chronic experimental monoarthritis by intra-articular injection of antigens into previously immunized **rabbits**.

Henderson et al. (1990) induced monoarticular arthritis in ovalbumin-sensitized rabbits by intraarticular injection of ovalbumin (antigen-induced arthritis) or in naive rabbits by injecting hyaluronic acid mixed with the polycation poly-D-lysine (polycation-induced arthritis).

Arner et al. (1995) compared the alterations in proteoglycan metabolism in antigen-induced arthritis and polycation-induced arthritis in rabbits and determined the involvement of interleukin-1 in the cartilage degradation that occurs in these models of rheumatoid arthritis.

Lewthwaite et al. (1995) studied the antifibrotic action of interleukin-1 receptor antagonist in antigen-induced monoarticular arthritis in New Zealand White rabbits.

Arthritis occurs in **pigs** due to infection with *Erysipelothrix rhusiopathiae* (Ajmal 1969). Experimental erysipelothrix infection in pigs can be used as a model for rheumatism research (Schulz et al. 1975a,b, 1977). Infections are established by oral or parenteral administration of standardized serotype B erysipelas strains.

Erysipelothrix arthritis could also produced in rats and **rabbits** (White et al. 1975; Glynn 1977).

Arthritis due to infection with *Mycoplasma synoviae* occurs naturally among domestic poultry (Olson et al. 1954, 1964). Arthritis in **chickens** after mycoplasma infection has been used as experimental model (Kerr and Olson 1970; Cullen 1977).

Experimental models of arthritis due to streptococcal infections have been proposed for various species: **mice** (Cayeux et al. 1966; Hook et al. 1960; Ohanian et al. 1969), **rats** (Jasmin 1967; Koga et al. 1973), **rabbits** (Cecil et al. 1939; Cook and Fincham 1966; Ginsburg et al. 1968, 1977; Norlin 1960; Shimizu et al. 1958; Stein et al. 1973), **pigs** (Roberts et al. 1968, 1969).

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I.2.2.9 Collagen type II induced arthritis in rats

PURPOSE AND RATIONALE

As reported by Trentham et al. (1977) intradermal injection of homologous or heterologous type II collagen in incomplete Freund's adjuvant results in an inflammatory polyarthritis in rats. The demonstration of antibodies to collagen in patients with rheumatic polyarthritis suggests that autoimmunity may contribute to the pathophysiology of synovitis and joint destruction. Because of the similarities of the symptoms in rats to human disease the test is considered to be useful to detect anti-inflammatory and immunosuppressive properties of test compounds.

PROCEDURE

Bovine type II collagen is prepared from nasal septum cartilage, which is cut into small fragments, frozen in liquid nitrogen, and pulverized in a freezer mill. Proteoglycans are extracted overnight by stirring 25 g of pulverized cartilage in 1 liter of 0.2 N NaOH. Following centrifugation at 20 000 *g* for 30 min, the residue is washed with 250 ml of absolute ethanol, the supernatant aspirated, and the residue vacuum dried. Hundred mg pepsin are added to 150 ml of 0.5 M acetic acid, after which 1.0 g of cartilage is added to reach a cartilage to pepsin ratio of 10 : 1 (w/w). The mixture is stirred 18 h at room temperature and centrifuged at 20 000 *g* for 1 h. Acid soluble collagen present in the supernatant is precipitated by adding NaCl to reach a final concentration of 0.9 M, followed by centrifugation at 20 000 *g* for 1 h. The precipitate from 1.0 g cartilage is dissolved in 100 ml 1.0 N NaCl/0.005 M Tris-HCl, pH 7.5, and stirred for 3 days. Then, the solution is dialyzed against 0.02 M Na_2HPO_4, pH 9.4, and the precipitate collected by centrifugation at 30 000 *g* for 1 h. The pellet is dissolved in 0.5 M acetic acid, dialyzed against 6 liters of 0.01 M acetic acid, and lyophilized. All procedures, unless otherwise stated are performed at 4 °C.

Test procedure. Collagen is dissolved in a concentration of 2.0 mg/ml in 0.1 M acetic acid overnight at 4 °C. This solution is added dropwise to an equal volume of chilled incomplete Freund's adjuvant. Six to 12 male Wistar rats with an initial weight of about 120 g are used for each group. On day 1, each rat receives a total of 0.5 mg collagen in 0.5 ml, equally divided, in 5 sites. All injections are intradermal, one at the base of each appendage and one in the nape of the neck. Seven days post-immunization, the animals receive identical booster injections. Control animals receive only the incomplete Freund's adjuvant diluted with 0.1 M acetic acid.

The volume of both hind paws is measured plethysmographically on day 20. To minimize the possibility of including animals with minimal transient disease, only animals with a paw volume of 1.8 ml or greater are used for further testing. From days 20–40, the animals receive the test compounds p.o. once a day. On day 41, the paw volumes are recorded again.

EVALUATION

The paw volumes of treated animals are recorded plethysmographically. The increase versus day 20 is calculated. The increase is compared with that of controls or animals treated with a standard drug. Otherwise, arthritic scores can be determined. Nonsteroidal anti-inflammatory drugs such as indomethacin in a dose of 2 mg/kg p.o. or phenylbutazone in a dose of 150 mg/kg p.o., but not acetylsalicylic acid in a dose of 50 mg/kg p.o. have been found to be active. Likewise, corticosteroids and immunosuppressives, but not D-penicillamine, were active.

CHRITICAL ASSESSMENT OF THE METHOD

Non-steroidal and steroidal anti-inflammatory compounds are detected by this method which, however, does not allow a separation between this two groups.

MODIFICATIONS OF THE METHOD

Hom et al. (1988), Takagishi et al. (1986, 1992), Cannon et al. (1990), Nemoto et al. (1992) and Carlson et al. (1992) described the effects of immunomodulating agents in collagen-induced arthritis in mice.

Wooley et al. (1993) investigated the anti-arthritic effect of recombinant human interleukin-1 receptor antagonist protein on type II collagen-induced arthritis and antigen-induced arthritis in mice.

Joosten et al. (1994) found an accelerated onset of collagen-induced arthritis in DBA_1 lac/J mice by remote inflammation.

Miesel et al. (1993, 1994a,b) studied the effects of an active center analogue of Cu_2Zn_2-superoxide dismutase in collagen type II-induced arthritis. Furthermore, the authors described a model potassium peroxochromate-induced inflammation in rats and mice. One to 3 µmol/kg K_3CrO_8 was administered by intraplantar application into the left hind-paws of anesthetized rats or mice. Arthritis index was assessed by a score system or the inflammatory response was quantified scintigraphically under a gamma camera by intravenous injection of 500 µCi $Na^{99m}TcO_4$.

From studies with a neutrophil elastase inhibitor Janusz and Durham (1997) concluded that of the destruction of the joints in rat collagen-induced arthritis is at least partially due to neutrophil elastase.

Kumar et al. (1997) compared the cellular mechanisms involved in the control of collagen II-induced

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I.2.2.10 Proteoglycan-induced progressive polyarthritis in mice

PURPOSE AND RATIONALE

Glant et al. (1987, 1992), Mikecz et al. (1987, 1990), Poole (1989) described a proteoglycan-induced progressive arthritis and spondylitis in BALB/c mice as an animal model displaying similarities to human rheumatoid arthritis and ankylosing spondylitis as indicated by clinical assessments, immunological parameters and histopathological studies of diarthrodial joints and spine.

PROCEDURE

High buoyant density cartilage proteoglycans are prepared from fetal and adult human, canine or bovine articular cartilages as well as from 1-week-old mouse epiphyseal cartilage. Fetal human articular cartilage proteoglycan digested with chondroitinase ABC (Hascall and Heinegård 1974) is used to induce arthritis in female BALB/c mice. The mice are sensitized by intraperitoneal injection of 100 µg of chrondroitinase ABC-treated proteoglycan in 100 µl of phosphate buffered saline, pH 7.2, and in Freund's complete adjuvant in a 1 : 1 emulsion. They are re-injected twice more with the antigen in incomplete Freund's adjuvant after one and three weeks. All BALB/c mice immunized with human articular cartilage proteoglycan develop arthritis in diarthrodial joints after the third antigen injection. Sera from mice with progressive polyarthritis are tested for antibodies to arthritogenic proteoglycans during weeks 12–18 of immunization. The limbs of all mice are examined daily to record clinical arthritic changes. Swelling and redness, as the first symptoms of arthritis, and the thickness (diameter) of the knee, ankle (intermalleolar diameter), wrist and

the dorsovo-lar thickness of the paw are recorded three times a week. The most objective joint diameter is the intermalleolar one. The animals are treated with test drug or vehicle for 12 weeks and serum samples taken by retroorbital puncture for determination of antibodies to proteo-glycans. Seven weeks later, the mice are sacrificed, limbs, tails and lumbar spine are fixed, decalcified and embedded in paraffin for histological examination.

EVALUATION

Mean values of intermalleolar diameter and antibody titers of treated and non-treated animals are compared by non-parametric statistics.

MODIFICATIONS OF THE METHOD

Stimpson and Schwab (1989) described a chronic remittent erosive arthritis in rats induced by bacterial peptidoglycan-polysaccharide structures.

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I.2.2.11 Experimental autoimmune thyroiditis

PURPOSE AND RATIONALE

Immunization of rats or mice with porcine thyroglobulin results in thyroiditis (Vladutiu 1971, 1983; McGregor et al. 1983; Hassman et al. 1985; Salamero et al. 1987; Fournier et al. 1990).

PROCEDURE

Crude porcine thyroglobulin (PTg) solution is emulsified in complete Freund's adjuvant in a 1 : 1 ratio. Female mice (6–8 weeks old) are primed with 50 µg PTg given s.c. into four or five sites of injection and are boosted 14 days later with the same dose of PTg (s.c.) emulsified in incomplete Freund's adjutant. The test compounds are administered from day 0 (at priming) until day 21. Mice are bled on day 21 and on day 28 after priming. The sera are tested for the levels of anti-PTg antibodies using an enzyme-linked immunosorbent assay (ELISA). On day 28, the animals are sacrificed and the thyroid glands prepared. Five-micrometer thick sections are stained with Masson-Goldner's trichrome solution.

EVALUATION

The histological severity of experimental autoimmune thyroiditis is graded as a function of mononuclear cell thyroid infiltration indices:

1. interstitial accumulation of inflammatory cells distributed between two ore more follicles.
2. one or two foci of inflammatory cells reaching at least the size of one follicle
3. 10 to 40% of the thyroid replaced by inflammatory cells.
4. more than 40% of the thyroid replaced by inflammatory cells.

Mean values of treated animals are compared with controls.

MODIFICATIONS OF THE METHOD

Castagliola et al. (1994) induced autoimmune thyroid disease in BALB/c mice by immunizing with the extracellular domain of the human TSH receptor expressed as a maltose-binding protein fusion in bacteria. This type of thyroiditis could be transferred to naive BALB/c and NOD mice (Castagliola et al. 1996).

Green et al. (1995) described a spontaneous model of autoimmune thyroiditis in MRL-lpr/lpr mice.

Furthermore, Green et al. (1996) induced thyroiditis in Lewis rats by immunization with thyroid extract and thyroglobulin. A reduction of the gap junction proteins connexin 43, connexin 32 and connexin 26 was found in diseased thyroid tissue.

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I.2.2.12 Coxsackievirus B3-induced myocarditis

PURPOSE AND RATIONALE

The effects of immunosuppressant drugs can be studied in the murine model of Coxsackie virus B-3 myocarditis.

PROCEDURE

Three week old male BALB/c mice are kept for 7 days before the experiment in a single, self-contained animal isolation unit to exclude pre-diseased animals. They are maintained in disposable, filter-topped cages and handled with gloves by gowned and masked personnel. The intraperitoneal route is used for injection of virus in a 0.5 ml volume.

The CVB3 virus strain is grown on either Hep-2 or VERO cells, aliquotted, and maintained at –70 °C until use. At the time of infection, seed virus is grown on either VERO or LLC-MK-2 cells with Dulbecco's modified Eagle medium, 12% fetal calf serum and gentamycin. Virus is harvested and adjusted to an inoculum of 1.75×10^7 plaque forming units/0.5 ml RPM-1640. The test drugs are given subcutaneously daily for 8 days. On day 8, the animals are sacrificed, the hearts rapidly removed and divided into two equal cross sections. The basal portion is snap frozen for isolation of virus and determination of drug level. The apical portion is fixed in 10% formalin, dehydrated and embedded in paraffin. Five mm sections are stained with hematoxylin-eosin and Masson's trichrome stains. The bases of the individual hearts are minced with a sterile scalpel, suspended in 1 ml RPMI-1640, and homogenized in a glass tissue grinder. The suspension is centrifuged at 8 000 *g* for 10 min at 4 ºC. Supernatants are harvested and frozen at –70 ºC until assay. Serial 10-fold dilutions of heart homogenates in minimum essential medium are layered on confluent, 72-h-old VERO cells, that had been grown in 96-well microtiter plates. Monolayers are checked daily for 7 days for presence or absence of virus and rate of cell destruction.

EVALUATION

The slides are examined by two observers blinded to the slide code, and inflammation and necrosis are quantitated.

MODIFICATIONS OF THE METHOD

Lane et al. (1991) showed that lipopolysaccharides promote CB3-induced myocarditis in otherwise resistant B10.A mice.

Beisel et al. (1991) identified a putative shared epitope between Cocksackie virus B4 and mouse alpha cardiac myosin heavy chain.

Gauntt et al. (1993) found that epitopes shared between Coxsackie virus B3 and normal heart tissue contribute to CVB3-induced myocarditis in mice.

Instead of Coxsackie virus B-3, Monrad et al. (1986) used encephalomyocarditis virus to induce experimental myocarditis in mice.

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I.2.2.13 Porcine cardiac myosin-induced autoimmune myocarditis in rats

PURPOSE AND RATIONALE

Pummerer et al. (1991), Inomata et al. (1995), Suzuki 1995; Dimitrijevic et al. (1998) described autoimmune myocarditis in rats induced by porcine cardiac myosin.

PROCEDURE

Male Sprague Dawley or Lewis rats at the age of 8 to 10 weeks are immunized with porcine cardiac myosin either purchased from Sigma (St. Louis, MO, USA) or prepared from the ventricular muscle of porcine hearts according to Murakami et al. (1976). The cardiac myosin fraction is dissolved in phosphate buffer at a concentration of 10 mg/ml. The antigen solution is emulsified with equal volume of complete Freund's adjuvant supplemented with heat-killed mycobacterium tuberculosis. Rats are injected subcutaneously into the foot pad with an immunizing dose of 5 mg of antigen in complete Freund's adjuvant/kg of body weight. Rats are injected intraperitoneally with test compounds either from day 0 to 6 (early treatment group) or from day 14 to 20 (late treatment group).

Immunized rats are sacrificed on days 8, 16, 21, and 34, respectively. Disease course and severity are analyzed by macroscopic findings of the hearts, heart weight/bodyweight ratio, as well as by histological and immunohistochemical analysis. Macroscopic findings are scored as follows: 0: normal finding; 1: presence of focal discolored area on the surface; 2: presence of diffuse discolored areas (Kodama et al. 1995).

The hearts are removed and weighted immediately after the rats are sacrificed, fixed in 10% buffered formalin and embedded in paraffin. Serial section (5 μm in thickness) are stained with hematoxylin-eosin. The severity of myocarditis is determined according the following scoring system: 0, no inflammation; 1: histological cross section infiltrated up to 5%; 2: 5–10% infiltrates/section; 3: 10–20% infiltrates/section; more than 20% infiltrates/section.

For immunohistochemical staining, heart samples are embedded in OCT compound (Miles, Elkhart, IN) and rapidly frozen. Cryostat sections are cut sequentially at 7 μm in thickness, mounted on glass slides and prepared for immunoperoxidase staining. Sections are fixed in cold acetone for 10 min and extensively washed in 0.1 M Tris buffer solution, pH 7.6. Murine monoclonal antibodies specific for different rat molecules are added at appropriate concentrations. After incubation at 4 °C over night and further buffer washes, the sections are incubated with peroxidase-conjugated anti-mouse immunoglobulins for 60 min. Peroxidase reaction is visualized with 0.05% diaminobenzidine in 0.01% H_2O_2 for 7–8 min. The color development is stopped by washing slides in running water. All samples are lightly counterstained with hematoxylin, mounted in gelatin/glycerol medium and assessed by light microscopy.

EVALUATION

Macroscopic and microscopic scores are expressed as mean values. Body weights, heart weights and heart weight/body weight ratio are expressed as mean ±SD. Student's *t*-test for paired samples is used for comparison data within groups in reference to time, while two-sample *t*-test is used for comparison data between groups.

MODIFICATIONS OF THE METHOD

Koyama et al. (1995) immunized Lewis rats with human cardiac myosin suspended in complete Freund's adjuvant and induced severe active myocarditis with acute and chronic heart failure. The baseline left ventricular pressure was significantly lower in the chronic phase group and peak *dP/dt* was significantly lower in both the acute phase group and the chronic phase group than in the respective controls. The animal model was recommended to study both acute heart failure related to acute myocarditis and chronic heart failure due to diffuse myocardial fibrosis.

Neu et al. (1990, 1991ab; Penninger et al. 1993) induced severe autoimmune myocarditis in some mouse strains by immunization with cardiac myosin in complete Freund's adjuvant.

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I.2.2.14 Experimental allergic encephalomyelitis

PURPOSE AND RATIONALE

Experimental allergic encephalomyelitis was first produced in laboratory animals by Rivers et al. in 1933. This pathological model is an immunologic disease arising from a delayed hypersensitivity reaction to nervous tissue. In many respects, the model resembles autoimmune diseases, especially demyelinating diseases, in man. The method is used for evaluation of immunosuppressive properties of drugs.

PROCEDURE

Preparation of the encephalitogen: 3 g of spinal cord from guinea pigs or rats are homogenized with 7.5 ml bidistilled water, 3.8 ml phenol and 7.5 ml complete Freund's adjuvant under cooling.

Groups of 6–12 male Wistar-Lewis rats with an initial body weight of 130–200 g are used. On day 0, experimental allergic encephalomyelitis is induced by subplantar injection of 0.1 ml of the encephalitogen into the left hind paw. An equal volume of *Bordetella pertussis* vaccine concentrate (200×10^9 organisms/ml) is injected into the same foot. From days 1–2, the animals receive the test compound or vehicle only or the standard drug by oral administration once a day. Body weights of the animals are recorded every second day. The clinical signs of experimental allergic encephalomyelitis consist of ataxia or paresis, i.e., grossly irregular gait and weakness of one or both hind legs followed by flaccid paralysis of the hindquarters, urinary incontinence, fecal impaction and abdominal wall flaccidity. Animals showing one of these clinical signs are considered positive for the purpose of evaluation.

EVALUATION

Starting from day 7, the severity of clinical signs and mortality are determined daily and scored according to the following scheme:

	score
per 20 g loss of weight	1
paralysis of the tail	1
paralysis of the hind paw	3
complete paralysis	5
death	6

Calculation of the results

The delay of onset of the paralytic symptoms is determined. The total score per day is recorded for treated and control groups. On the day of maximal clinical symptoms occurring among control animals, the total scores of the treated groups is compared to the total score of the control group. The percentage change is evaluated.

Doses of 0.5 mg/kg p.o. methotrexate, 1 mg/kg p.o. hydrocortisone, 2.5 mg/kg p.o. cyclophosphamide were found to be active, whereas non-steroidal anti-inflammatory compounds were inactive.

CHRITICAL ASSESSMENT OF THE METHOD

The model of experimental allergic encephalomyelitis in rats is suitable to distinguish between immunosuppressive and anti-inflammatory drugs.

MODIFICATIONS OF THE METHOD

The phosphodiesterase inhibitor pentoxifylline was found to prevent induction of experimental autoimmune encephalomyelitis in Lewis rats (Rott et al. 1993).

Martin and Near (1995) studied the protective effect of the interleukin-1 antagonist IL-1ra on experimental allergic encephalomyelitis in Lewis rats.

Experimental autoimmune encephalomyelitis in different strains of mice was described by Heremans et al. (1996), Glabinski et al. (1997), Liblau et al. (1997).

Baker et al. (1990, 1991, 2000) induced experimental allergic encephalomyelitis in Biozzi AB/H mice by sensitization with 1 mg of mouse spinal cord homogenate emulsified in Freund's complete adjuvant on days 0 and 7. The disease is characterized by relapsing-remitting episodes similar to multiple sclerosis in human beings. Biozzi AB/H mice also develop spasticity and tremor which can be antagonized by cannabinoids.

A chronic relapsing-remitting form of experimental autoimmune encephalomyelitis was induced in the common marmoset Callithrix jacchus following a single immunization with human white matter by Massacesi et al. (1995), Genain and Hauser (1997) and recommended as a new model for multiple sclerosis.

Experimental allergic neuritis in several animal species has been described by Waksman and Adams 1955, 1956; King et al. 1983; McCombe et al. 1990; Nakayasu et al. 1990). This disorder has been considered to show similarities to the Guillain-Barré syndrome in man. The demyelating process initiated by the injected antigens is a lymphocyte-mediated reaction in which activated macrophages strip myelin off the axons. Hartung et al. (1987) described the adoptive transfer experimental autoimmune neuritis in Lewis rats by injection of P2-reactive T lymphocyte cell lines.

Mix et al. (1992) studied the effect of stilbene-type anion channel blockers on the immune response during experimental allergic neuritis induced by bovine peripheral myelin.

Kojima et al. (1994) investigated the pathogenic potential of autoimmune T cell responses to nonmyelin autoantigens in the Lewis rat using the astrocyte-derived calcium-binding protein S100β, as a model nonmyelin autoantigen. In contrast to the experimental autoimmune encephalomyelitis induced by the adoptive transfer of myelin basic protein-specific T line cells, S100β-specific T cell transfer induced intense inflammation not only in the spinal cord but also throughout the entire CNS and also in the uvea and retina of the eye.

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I.2.2.15 Acute graft versus host disease (GVHD) in rats

PURPOSE AND RATIONALE

The intravenous injection of a mixture of parental splenocytes into healthy inbred F_1-rats results in graft-versus-host (GVH) induced immune abnormalities. This is due to T-lymphocytes in the donor inoculum that recognize the major histocompatibility alloantigens expressed by the F_1-animals. The host F_1 T-cells are genetically unable to recognize antigens of the parental donor as foreign, thus the response involves only donor recognition of host and not host recognition of donor. The ensuing immune abnormalities lead to clinical symptoms of an acute, lethal GVH-disease (GVHD), i.e. profound immunodeficiency, anemia, hypogammaglobulinemia and runting.

PROCEDURE

Three to 4 month old, male F_1-hybrid rats of the inbred strains Lewis (Rt-1 l) and Brown Norway (BN, Rt-1n) (Zentralinstitut für Versuchstierkunde Hannover Germany) are used as hosts for cell grafts from the Lewis parental strain. The bone marrow cells are obtained by flushing hind femur bone shafts with culture medium. These cells are then pooled together with spleen cells (ratio 2 bones: 1 spleen). The cell viability, determined by trypan exclusion, has to be more than 90%. Each recipient is injected with about 40×10^7 cells in a 1.5 ml-suspension volume. The route of injection is the penis vein, allowing an optimal control of correct intravenous application.

Prophylactic drug application

For this experiment, 2 groups of 6 F_1-hybrids each are injected with the above mentioned bone marrow/spleen cell suspension. One group receives the test drug orally and daily until the end of the experiment, homogeneously suspended in 1% carboxy-methylcellulose (CMC) solution. The other group receives CMC alone and, thus, serving as the GVHD control group. The experiment is terminated two weeks after disease induction i.e. 1 week after the first appearance of GVHD-symptoms. All animals are sacrificed and clinical aspects documented, spleens weighed, histology of the skin, liver, spleen and lymph-nodes performed and organs photographed.

Therapeutic drug application

In this experiment, rats are separated into four groups treatment begins with the first sign of GVHD-symptoms (beginning of the second week). Because of the expected, greater therapeutic difficulty, the daily dose of the test drug has to be doubled, again for 2 weeks duration.

The experiment is terminated either by sacrificing those rats that are too sick to be able to move around the cage, or at the end of the 4 week observation period, regardless of the clinical condition of the animals. The clinical-chemical parameters are determined by routine procedures conducted with a HITACHI autotechnicon.

EVALUATION

The tested parameters of therapeutic success or disease, respectively, are survival rate (%), spleen weight (g), and body weight (g) as well as clinical-chemical parameters (bilirubin, alkaline phosphatase, creatinine, white cell count) after 2 and 3 weeks.

MODIFICATIONS OF THE METHOD

Gelpi et al. (1994) established a chronic graft vs host disease in (C5BL/10 x DBA/2)F_1 mice with an injection of lymphoid cells from the parent DBA/2 strain. Most of the animals developed antibodies against transfer RNA/protein particles.

Mosier et al. (1988) reported transplantation of human peripheral blood lymphocytes (PBL) into severe combined immunodeficient (scid) mice to construct hu-PBL-scid mice. Kim et al. (1997) suggested these mice for routine immunotoxicity investigations using lymph nodes of intestines as the lymphocyte sources.

Ford et al. (1970), Schorlemmer et al. (1997, 1998) used the popliteal lymph node assay to study the local graft vs. host reaction. The test is based on the enlargement of the draining popliteal lymph nodes as a result of injecting immuno-competent cells (1×10^8 parental Lewis spleen cells) into the hind foot pad of Lewis × Brown-Norway F1 recipients. The reaction is measured at day 6 after challenge as a gain in lymph node weights.

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I.2.2.16 Influence on SLE-like disorder in MRL/lpr mice

PURPOSE AND RATIONALE

Systemic lupus erythematosus (SLE) is an autoimmune disease in man that affects multiple body organs and is characterized by the development of certain types of self antigens. Primarily, the antibodies formed against double-stranded DNA (dsDNA), the most prevalent in this ailment, complex together and, with complement, deposit in the small blood vessels, leading to widespread vasculitis. MRL Mpf lpr/lpr (MRL/lpr)-mice spontaneously develop a severe disease with many symptoms very similar to human SLE, i.e. hypergammaglobulinemia, and glomerulonephritis (Theofilopoulos and Dixon 1981).

PROCEDURE

Female MRL/lpr-mice (originally from Jackson Laboratories, USA), displaying distinct symptoms of SLE (between 12 and 13 weeks of age) are randomized and divided into groups of 12 animals each. At this age, the animals have already clinical manifestations of the SLE-like illness, as determined by the disease index, but have not yet developed proteinuria. Animals with

early symptoms of disease are treated with various drugs, e.g., leflunomide, cyclosporin A, azathioprine, cyclophosphamide or prednisolone for 11 weeks and the survival rate and disease index of these animals are followed for 24 weeks. The disease index and urine protein level are determined once weekly.

Disease index

The subsequent clinical parameters are taken into consideration:

1. Ears: reddening of the skin, deterioration of the pinna.
2. Nose: loss of hair, wasting of the skin.
3. Lymph nodes: detection of swollen lymph nodes on any part of the body, especially neck and extremities.
4. Fur: general condition of fur (e.g. shabby, mangy, etc.), loss of hair.
5. Skin: inflammation of the skin, scab and/ or granuloma formation.
6. Eyes: exophthalmos, deterioration due to inflammation, tumor formation around the eye, swelling of the eyelid with eventual closure of the eye.
7. Paws: reddening of the skin, swelling of the paw.

EVALUATION

A score for each of the above described parameters is given according to the severity of the symptoms as follows:

Points for clinical index

Involvement	*detectable*	*moderate*	*severe*
Ears (each)	0.5	1.0	1.5
Nose	1.0	2.0	3.0
Lymph node (each)	1.0	2.0	3.0
Fur	1.0	2.0	3.0
Skin	1.0	2.0	3.0
Eyes (each)	1.0	2.0	3.0
Paws (each)	0.5	1.0	1.5

Body weight (one point for 5 g difference from week to week)

The determination of the disease index is performed, weekly, by the same individual, but without knowledge of the group being evaluated. The points, for each animal, are registered and the total score, of each group, summarized. The average score for the group is calculated, significance between the experimental group and the untreated diseased group is determined using the Student *t*-test.

Proteinuria

Pooled urine is collected from each experimental group and the amount of protein in the urine is calculated.

MODIFICATIONS OF THE METHOD

In addition to a lupus-like syndrome and massive T cell proliferation. MRL-1pr/1pr (MRL/1) mice develop an arthritic process very similar serologically and histologically to human rheumatoid arthritis. Boissier et al. (1989) found that in these animals mouse type II collagen is antigenic, but not arthritogenic.

Holmdahl et al. (1991) studied the involvement of macrophages and dendritic cells in synovial inflammation of collagen induced arthritis in DBA/1 mice and spontaneous arthritis in MRL/Lpr mice.

Rordorf-Adam et al. (1985) used serum amyloid P component and autoimmune parameters in the assessment of arthritis in MRL/lpr/lpr mice.

Furukawa et al. (1996) studied the autoimmune disease-prone genetic background in relation to Fas-defect in MRL/lpr mice.

Kanno et al. (1992) found spontaneous development of pancreatitis in the MRL/Mp strain of mice.

Kusakari et al. (1992) compared hearing acuity and inner ear disorders of MRL/lpr mice with those of BALB/c mice and found a significantly higher auditory brain stem response threshold. They recommended this as a model of sensorineuronal hearing loss.

Bundick and Eady (1992) investigated the effects of an immunosuppressive agent on the development of spontaneous lupus disease in female NZBW F_1 hybrid mice.

Walker et al. (1996) reported a powerful suppressive effect of testosterone on the autoimmune disease analogous to systemic lupus erythematodes spontaneously developed by F1-hybrids of New Zealand Black (NZB) × New Zealand White (NZW) mice. A model was developed in which NZB dams carrying NZB/NZW fetuses were treated with testosterone in a dose adequate to masculinize the external genitalia in female fetuses.

Zoja et al. (1998) investigated bindarit, a compound devoid of immunosuppressive properties, in NZB/W F1 hybrid mice developing an immune complex glomerulonephritis with proteinuria and progression to renal insufficiency.

Kiberd and Stadnyk (1995) studied the role of endogenous interleukin-1 in established lupus nephritis in MRL lpr/lpr mice by administration of the IL-1 receptor antagonist IL-1ra.

Gleichmann et al. (1982), Schorlemmer et al. (1997) induced a systemic lupus erythematodes-like disease in mice by abnormal T- and B-cell cooperation. A chronic graft versus host reaction with the pathologic symptoms of severe glomerulonephritis is induced in B6D2 (C5Bl/6 × DBA/2) F1 hybrid mice receiving four i.v. injections (one per week) of 1×10^8 parental lymphoid spleen cells from DBA/2 donors. The inoculation of splenocytes into the BDF1 hybrid mice results in the

development of a chronic GvH reaction with lymphoid hyperplasia, autoantibody production and immune-complex glomerulonephritis.

Chan et al. (1995) described ocular changes occurring in mice with experimental lupus erythematodes. The ocular disease is characterized by bilateral subacute and chronic inflammation of the eyelids (blepharitis) and hypertrophic Meibomian glands. The severity of the ocular changes is strain dependent. The authors recommend this experimental eye disease as an animal model for chronic blepharitis in humans.

The changes of lacrimal and salivary glands found in MRL/lpr mice and other mouse strains with autoimmune disorders were also regarded as model of Sjögren's syndrome in human (Sullivan and Edwards 1997; Toda et al. 1999).

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I.2.2.17 Prevention of experimentally induced myasthenia gravis in rats

PURPOSE AND RATIONALE

Myasthenia gravis is an organ specific autoimmune disease in man that results in skeletal muscles weakness. Typically, the sufferer has drooping eyelids, a blank facial expression, and weak, hesitant speech. This is due to the formation of autoantibodies against the nicotinic acetylcholine receptor (AChR). The formation of autoantibodies to acetylcholine's receptor leads to a gradual destruction of the receptors in skeletal muscles that receive nerve impulses and initiate muscle contractions. As a result, affected muscles fail to respond or react only weakly to nerve signals.

Experimental myasthenia gravis (EMG) can be induced in rats by injecting them with heterologous

AChR, or with recombinant α subunits (two) of the AChR (portion of the AChR to which acetylcholine mainly binds) (Lennon et al. 1991). The animals display symptoms of myasthenia (electrophysiological evidence of altered neuromuscular function) and detectable antireceptor antibodies. The two α-subunits are plausible targets of pathogenic autoantibodies because they would allow mechanisms that depend on cross-linking, complement-mediated lysis, or hindrance of acetylcholine binding to the receptor (Schwartz 1993). The severity of the disease can vary, but most animals display, at the very least, a weakness and fatigability of foot grip. The disease gradually leads to abnormal gait and eventually the inability of the animals to walk or even right themselves.

PROCEDURE

Female rats of AO strain, 6–10 weeks old, are used. Three groups of rats are included in the experiment:

1. immunized with acetylcholine receptor (AChR)-protein and treated with test drug;
2. immunized with AChR-protein without drug, and
3. nonimmunized, nontreated control rats. The test drug is applied per os daily. First dose is administered on the day of immunization and the last on the day of sacrifice.

Immunization with AChR-protein

AChR-protein isolated from Torpedo marmorata is emulsified with complete Freund's adjuvant and 100 µg/rat is injected intradermally in the hind foot pad. As additional adjuvant, 2.6×10^{10} *Bordetella pertussis* microorganisms is administered simultaneously by intramuscular injection in the hind leg.

Antibody determination

Anti-AChR-protein antibodies are measured by enzyme linked immunosorbent assay (ELISA) as described by Norcross et al. (1980). AChR-protein is diluted to a final concentration of 2.5 µg/ml in 0.05 M carbonate buffer, pH 9.6. Two hundred ml of this solution is placed in each well of a microtitration plate (Flow Laboratories Inc.). After an over night incubation at 4 °C, the plates are washed thoroughly with 0.01 M phosphate buffered saline (PBS) solution containing 0.05% Tween-20 (Sigma) subsequently referred to as PBS/T. Sera from all groups of rats are serially diluted in PBS/T and 200 µl is added to each micron well except in the background row (control row) and incubated at 4 °C for 2 h. After washing, 200 µl of 1:1 000 diluted peroxidase conjugated goat antirat immunoglobulin (Sera Lab. Sussex, England) in PBS/T are added to the micron wells and incubated for an additional 60 min at 4 °C. After plates are washed, 200 µl of substrate-citrate buffer and 0.2 µl of 10% H_2O_2 are added and then incubated in the dark at room temperature for 30 min. The reaction is stopped by addition of 50 µl of 2M H_2SO_4 and the OD determined by using Titert Multiscan.

Two-color flow cytometry

Thymic cell suspensions are obtained by mincing tissue and passing it through 80 mm stainless mesh. After being washed three times in PBS, the cells are resuspended in PBS at a cell density of 10^7 viable cells/ml. The cell viability is determined by the trypan blue exclusion test. Erythrocytes are removed by addition of ammonium chloride. Cell staining and flow cytometric analyses are done as described by Itoyama et al. (1989). Thymocyte subsets expressing CD4 and/or CD8 molecules are defined by staining with monoclonal antibodies obtained from Serotec, Oxford, England: phycoerythrin (PE)-conjugated anti-W3/25 (CD4) and fluoresceinisothiocyanate (FITC)-conjugated anti-MRC OX8 (CD8). Two $\times 10^5$ to 1×10^6 cells suspended in 100 ml of PBS are exposed sequentially for 30 min to FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 monoclonal antibodies. Isotype matched control monoclonal antibodies are used to prove the specificity of binding. Cell analysis is performed using FACScan flow cytometer from Becton Dickinson. One $\times 10^4$ events per sample are analyzed by Consort 30 and Lysis software. All data are collected and displayed on a log scale of increasing green and orange fluorescence intensity. This is presented as two-dimensional contour maps and as percentage of thymocytes by integrating counts in selected areas of the contour plots.

Stereologic analysis of thymuses

Thymuses of animals of all groups are prepared for light microscopic analysis. For this purpose thymus tissue is fixed in Carnoy's solution, embedded in paraffin and 3–5 µm thin sections are stained with hematoxylin and eosin. Cortex and medulla are analyzed stereologically using the point counting method described by Weible (1963). Volume density (*Vv*) of the examined structures is determined by the following equation: $Vv=Pi/Pt$, where *Pi* represents the number of points of the examined structure, and *Pt* the total number of points. *Vv* refers to the volume fraction i.e. volume of a feature per unit test volume (Williams 1981).

EVALUATION

EMG is evaluated clinically by daily examination of muscle weakness and scored as follows:

\+ = weakness of grip with fatigability;
++ = abnormality of gait;
+++ = inability to walking and righting.

Immediately after appearance of clinical signs of EMG, rats are sacrificed, blood and thymuses are taken for determination of anti-AChR-protein antibodies, and histological analysis of thymuses and thymocyte subsets, respectively.

Statistical analysis of data is performed by Student's *t*-test (data of stereological analysis) and Mann-Whitney *U*-test (Results of flow cytometric analysis of thymocyte subsets).

MODIFICATIONS OF THE METHOD

McIntosh and Drachman (1987) described an *in vitro* suppressor assay using responder cells from the lymph nodes of Lewis rats immunized sc. with acetylcholine receptors emulsified in complete Freund's adjuvant and suppressor cells from spleens of rats immunized i.p. with acetylcholine receptors absorbed on bentonite. Antibodies were determined after stimulation with acetylcholine receptors from co-cultures of responder cells and putative suppressor cells treated previously with an immunosuppressant.

Araga and Blalock (1994) developed a method of altering B-cell-mediated autoimmune diseases by induction of anti-idiotypic antibodies by immunization with complementary peptides. A peptide encoded by RNA complementary to RNA for the Torpedo acetylcholine receptor main immunogenic region, AChR 67-16, was tested in the Lewis rat model of experimental auto-immune myasthenia gravis.

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I.2.2.18 Glomerulonephritis induced by antibasement membrane antibody in rats

PURPOSE AND RATIONALE

Masugi nephritis and other nephritis models of immunological origin in rats have been used for evaluation of immunosuppressive activity (Heymann et al. 1959; Shibata et al. 1966; Ito et al. 1983; Thoenes et al. 1989; Ogawa et al. 1990, 1991).

PROCEDURE

Preparation of rabbit antiserum against rat glomerular basement membrane

Glomeruli are separated from the homogenate of rat renal cortex by successive use of 3 metal sieves (150-, 180-, and 200-mesh). The basement membrane fraction is obtained by centrifugation and ultrasonic disruption. It is then digested with trypsin, dialyzed, and lyophilized. The resultant substance is employed as antigen. An emulsion of 1 mg of the antigen in 0.2 ml saline with 0.2 ml of complete Freund's adjuvant is injected intracutaneously into white rabbits once a week for 6 weeks. One week later, production of the antibasement membrane antibody is confirmed in guinea pigs by the passive cutaneous anaphylaxis test. Blood is collected from the carotid artery, incubated at 56 °C for 30 min to inactivate components of the complement, and stored at –20 °C until use.

Induction of glomerulonephritis in rats

Male Sprague-Dawley rats weighing about 300 g are injected with 0.5 ml of the rabbit antiserum via the tail vein. On the following day, they are further injected subcutaneously with an emulsion (0.25 ml) of physiological saline solution containing 5 mg of rabbit gamma-globulin in an identical volume of complete Freund's adjuvant.

Treatment

The rat antibasement antibody is injected 5 days before the start of administration of the test compound. Before the first dose, urinary total protein is determined and rats with nephritis are so assigned as to provide almost equal distribution of severity of the disease per group. The test compounds are administered orally for 14 days. The urine is collected at 7 and 14 days of treatment. After 14 days, the animals are sacrificed, blood is collected and thymus and kidneys are removed. Histopathological and immunohistochemical studies are performed in kidney tissue.

EVALUATION

Scores are given for **microscopic findings in the glomeruli:**

- cell proliferation in glomeruli
- PAS-positive granules in the epithelium of glomeruli
- fibrin deposits in Bowman's space
- adhesion to Bowman's capsule

and in **the tubuli:**

- hyaline cast
- dilation of tubuli

as well as for **immunofluorescence findings** for rat IgG, rat C3, and rabbit IgG.

Furthermore, total urinary protein, plasma total cholesterol, plasma fibrinogen, thymus/body weight ratio are compared between drug treated animals and controls by statistical means.

MODIFICATIONS OF THE METHOD

Lan et al. (1995) investigated the pathogenic role of interleukin-1 in the progression of established rat crescentic glomerulonephritis by administration of the interleukin-1 receptor antagonist IL-1ra.

Giménez et al. (1987), Thoenes et al. (1987) induced autoimmune tubulointerstitial nephritis in the brown Norway rat by injection of bovine tubular basement membrane.

Development of a systemic T-lymphocyte dependent autoimmune syndrome in brown Norway rats including glomerulonephritis with high proteinuria was induced with mercuric chloride by Baran et al. (1986), Aten et al. (1988), Lillevang et al. (1992).

Kokui et al. (1992) induced nephrosis with proteinuria in rats by intraperitoneal injection of puromycin aminonucleoside.

Lundstrom et al. (1993) studied the Heymann nephritis antigenic complex using a rat yolk sac carcinoma cell line that expresses glycoprotein 330, the main antigen in this autoimmune disease.

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I.2.2.19 Auto-immune uveitis in rats

PURPOSE AND RATIONALE

The mechanisms of certain types of uveitis have been studied in animals implicating autoimmunity. Uveitis has been produced in guinea pigs following injections with homologous uveal tissue.

The method is described in detail in the Sect. O.7.3.

I.2.2.20 Inhibition of allogenic transplant rejection

PURPOSE AND RATIONALE

Transplantation of allogenic organs to recipients results in rejection of the transplants. This effect can be suppressed or delayed by immunosuppressive agents. Various organs are used for allogenic transplantation in animal experiments, such as skin pieces (Schorlemmer et al. 1993), kidney (Lee 1967; Küchle et al. 1991), rat heart, rat small intestine (Xiao et al. 1944) and corneal buttons (Coupland et al. 1994). The immunosuppressive activity can be evaluated either by using a major histocompatibility complex variant strain combination or a strong allogenic system.

PROCEDURE

For **skin transplantation** male animals of inbred strains of Fischer (F334), Lewis (LEW), Brown Norway (BN), Dark Agouti (DA) rats are used. Rat tail skin (donor) is cut into square pieces of 0.5 to 1.0 cm and transplanted to the tails of recipient rats. Rejection is defined as the day when the skin graft is of red-brown color and hard consistency. As strain combination with a major histocompatibility variant, transplantation from LEW to F334 is performed. Using a strong allogenic system, the high responder DA to LEW donor recipient combination is used. The immunosuppressive agents, e.g., cyclosporine or leflunomide are given orally up to 20 days. Ten animals are used for each group.

EVALUATION

The mean values of rejection time of treated groups are compared statistically with vehicle treated controls using Student's *t*-test or the Mann Whitney U-test.

MODIFICATIONS OF THE METHOD

Schorlemmer and Kurrle (1997) used Lewis (LEW, Rtl*l) rats as receivers and Balb/c mice as donors in a xenotransplantation model of mouse-to-rat skin grafts. Rejection was defined as the day when the skin graft turned red-brown and became hard. For quantification of xeno-specific IgM and IgG antibody titers, the test sera (dilution 1 : 10) were incubated with 1×10^6 purified T-cells (by sheep anti-mouse dynabeads, Deutsche Dynal GmbH, Hamburg, Germany) from Balb/c donor spleens for 30 min at 4 °C. The cells were washed 3 times with phosphate buffered saline (pH 7.2) and then stained for IgG or IgM xenoantibodies; 50 µl of either FITC-conjugated goat antibodies, specific for the Fc-portion of rat IgG or specific for the µ-chain of rat IgM were added. After 30 min at 4 °C the cells were washed twice and analyzed by flow cytometry.

Techniques for transplantation of several organs have been elaborated.

For **kidney transplantation** male rats, 5–7 months of age, are used as donors and recipients for the orthotopic right kidney transplantation as described by Lee (1967) with a modification of ureter-ureter anastomosis (Thoenes et al. 1974). Because bilateral nephrectomy is performed at transplantation, animal survival is dependent upon the allograft's function. All rats that do not excrete urine on the first postoperative day are excluded from further studies. As a control concerning long survival, syngeneically transplanted rats are maintained up to 300 days.

Engelbrecht et al. (1992) described a new rapid technique for renal transplantation in the rat. The method

combines a special sleeve anastomotic technique for the renal artery, convential end-to end anastomosis of the renal vein, and implantation of the ureter into the bladder.

A porcine renal transplant model has been used by Almond et al. (1992).

Peters et al. (1993) reviewed the therapeutic potential of tacrolimus in renal and hepatic transplantation.

For studying **heart transplantation**, heterotopic implantation of hearts from BN to LEW rats is performed (Williams et al. 1993). The diagnosis of rejection is established once the palpable cardiac allograft impulse ceases. Further studies with rat cardiac allografts have been performed by Hancock et al. (1990). The Fischer 344 rat (donor)/Long Evans rat (recipient) combination was used by Kahn et al. (1991). Walpoth et al. (1993) used magnetic resonance spectroscopy for assessing myocardial rejection in the transplanted rat heart.

Shiraishi et al. (1995) evaluated the effectiveness of the interleukin-1 receptor antagonist IL-1ra in the immune and inflammatory responses to rat heart allografts.

Cardiac transplantation between inbred rat strains that differ for weak histocompatibility antigens is associated with the development of arteriosclerosis in arteries of the donor graft myocardium (Carmer et al. 1990; Adams et al. 1992).

A heterotopic rat **infection heart-transplant model** was described by Kobayashi et al. (1993).

The **hamster to rat cardiac xenograft** model has been used by several authors (de Masi et al. 1990; Steinbrüchel et al. 1991; van den Bogaerde et al. 1991; Woo et al. 1993; Fujino et al. 1994; Schuurman et al. 1994). The hearts from Syrian hamsters were implanted heterotopically in male Lewis rats, with anastomoses between the infrarenal abdominal aorta and inferior vena cava of the recipient and the donor aorta and right pulmonary artery, respectively.

Primate cardiac xenografts were performed by McManus et al. (1993) using cynomolgus monkeys (*Macaca fascicularis*) as donors and baboons (*Papio anubis*) as recipients.

Chronic rejection of rat **aortic allograft** was studied by Mennander et al. (1991). Administration of cyclosporin induced accelerated allograft arteriosclerosis.

Heterotopic transplantation of small intestine has been performed from BN to LEW rats. The mesenteric venous drainage is reconstructed either via the vena cava or the portal vein (Xiao et al. 1994). An isolated Thiry-Vella-loop was prepared by Xia and Kirkman (1990). Kellnar et al. (1990) described allogenic transplantation of fetal rat intestine with anastomosis to the normal bowel of the host. Langrehr et al. (1991) investigated under which circumstances graft versus host disease occurs following fully allogenic small bowel transplantation in the rat. Kirsch et al. (1991) studied the extent to which intestinal transplants in rats undergo functional and morphologic compensation.

Liver transplantation procedure has been described by Svensson et al. (1995) allowing measurement of bile secretion.

Orthotopic left **lung transplantation** was performed in inbred rats by Katayama et al. (1991).

Tracheal allografts were implanted into the abdomen of recipient rats (Davreux et al. 1993).

In vivo electrophysiology of rat **peripheral nerve transplants** was studied by Yu et al. (1990). A sciatic-tibial nerve graft was harvested from the donor rat between the sciatic notch and the ankle. In the recipient, the tibial nerve and the sural nerve were resected. The nerve graft was placed along the natural course of the native tibial nerve. Nerve repair was performed using standard end-to-end epineural microsuture technique.

A model of neurovascularized rectus femoris **muscle transplantation** in rats was established by Muramatsu et al. (1994).

The orthotopic **transplantation of vascularized skeletal allografts** (rat distal femur and surrounding muscular cuff) has been described by Lee et al. (1995).

Long-term surviving of **limb allografts** in rats was studied by Kuroki et al. (1991). The donor and recipient limbs were prepared simultaneously by amputation at midfemur. The donor limb was fixed orthotopically by Kirschner wire. The donor and recipient femoral arteries, veins and sciatic nerves were anastomosed using a microsurgical technique.

For **cornea transplantation** Brown Norway rats ($RT1^{1xn}$) serve as donors and Lewis rats ($RT1^{1}$) as recipients (Coupland et al. 1994). Both the donor and recipient rats are anesthetized with xylazine hydrochloride and ketamine hydrochloride. Twenty min prior to surgery the recipient rats also receive 0.5 mg/kg atropine sc. and phenylephrine hydrochloride 5% eyedrops. Under sterile conditions and using an operation microscope, two donor corneal buttons (3.5 mm) are harvested from the donor rat using a trephine and curved Castroviejo scissors. The donor animals are then sac-

rificed by ether inhalation. The left eyes of the recipient rats are prepared by removing a central 3.0-mm button using a trephine and curved Castroviejo scissors. A drop of sterile methylcellulose (1%) is placed over the 3.0-mm corneal opening before the donor cornea is fixed with 10 interrupted sutures. The anterior chamber is not re-established following surgery. Prior to closure of the eyelids with 3 or 4 interrupted sutures, Polyspectran® eyelid gel is placed over the operated eye. Forty-eight hours following surgery, the eyelid sutures are removed, allowing for the first time assessment of the cornea on the slit-lamp microscope. Slit-lamp evaluations are performed every 2–3 days under i.m. anesthesia with keta-mine, with assessment of the cornea by scoring graft opacity, edema and vascularization.

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Chapter J
Activity on the gastrointestinal tract[1]

[1] Review by M. Bickel, contributions to the first edition by A. W. Herling.

J.1
Salivary glands

J.1.0.1
Measurement of salivation

PURPOSE AND RATIONALE

Symptoms of several human diseases are manifested as increased salivation (e.g., Parkinson's disease) or decreased salivation (e.g., xerosis). Studies to find and to evaluate sialagogues, such as substance P and its synthetic derivatives, as well as to search for salivation inhibitors are necessary. Saliva excretion is greatly influenced by anesthetics. Wagner et al. (1991) proposed a simple method to study saliva secretion in conscious rats and to evaluate sialagogues and sialagogue antagonists.

PROCEDURE

Fed, male Sprague-Dawley rats (200–300 g) are weighed and distributed randomly into groups of 6 animals. Conscious rats are injected i.v., via the lateral tail vein, with either the vehicle or the sialagogue, e.g., sub-stance P (0.3–3 µg/kg in 1 ml saline/kg). The rat's oral cavity is swabbed immediately after i.v. injection by placing and holding a pre-weighed, absorbent foam cube (e.g., 5/16", Texwipe Company, Upper Saddle River, NJ) sublingually for 10 s using a Triceps foam pencil (Texwipe Company, Upper Saddle River, NJ). Conscious rats are restrained during the 10 s collection period by gently holding the animal and opening the mouth using a plastic coated snare, which is looped around the maxillary incisors and drawn back over the animal's head and the hand holding the rat, drawn around in front of the rat and looped around the mandibular incisors. Gentle pressure on the snare opens the rat's mouth allowing the placement of the absorbent cube. Foam cubes are re-weighed immediately after use. The difference between the initial weight of the cube and the weight of the cube after use represents saliva secreted.

EVALUATION

Data are analyzed with Dunnett's *t*-test that compares several treated groups with a control group. Regression analysis is used to determine dose response and relative potency.

MODIFICATIONS OF THE METHOD

Martinez et al. (1978, 1981) inserted appropriate plastic cannulae into the main excretory ducts of the two submandibular glands in **rats**.

Giuliani et al. (1988) studied the relative contributions of various neurokinin receptors (NK-1, NK-2, NK-3) to the sialogogic response after i.v. application in urethane-anesthetized rats.

Direct cannulation of the glandular duct with polyethylene tubing was performed by Bodner et al. (1983) and Kohn et al. (1992).

Bianciotti et al. (1994, 1996) cannulated the ducts of both the submaxillary and parotid glands in male Wistar rats anesthetized with 10% ethyl urethane. No basal flow of saliva was observed from either gland, however dose-response curves could be established after intravenous injection of sialogogic agents, such as methacholine (0.3 to 10.0 µg/kg), norepinephrine (3 to 60 µg/kg), isoproterenol (1 to 30 µg/kg), methoxamine (30 to 300 µg/kg), substance P (0.3 to 10.0 µg/kg). Atrial natriuretic factor enhanced the salivary response to methacholine, methoxamine and substance P.

Lohinai et al. (1997) determined salivary amylase secretion in conscious rats. Under ether anesthesia a catheter was introduced into the esophagus for salivary juice collection and a cannula was inserted into the jugular vein for infusions. After post-anesthesia recovery, submaximal carbachol infusion was given as a background to obtain steady secretion because of the low basal secretory rate. After application of drugs, volume and amylase were determined in saliva samples collected for 30 min.

Iwabuchi et al. (1994) studied salivary secretion after administration of a muscarinic agonist in MRL/lpr **mice**. Saliva were collected from the floor of the mouth of anesthetized rats with a capillary micropipette every 5 min for 60 min.

A method for the quantitative comparison of atropine substitutes on the salivary secretion of the **cat** has been published by Bülbring and Dawes (1945). Cats anesthetized with pentobarbitone were used. A cannula is tied into Wharton's duct and attached to a bottle containing tap-water. The tap-water, displaced by the saliva, passes out of the bottle through a tube which actuates a drop timer.

Ekström et al. (1994) used morphometric analyses to study the parotid acinar degranulation in cats after stimulation of the parasympathetic auriculo-temporal nerve.

Izumi and Karita (1994, 1995a,b) investigated the secretory and vasodilator effects of nerve stimulation in the submandibular gland of cats. Cats of either sex were anesthetized with ketamine and a mixture of chloralose and urethane, paralyzed by intravenous injection of pancuronium bromide and artificially ventilated. Blood flow changes in the submaxillary glands and lips of the cats were measured using a laser-Doppler flowmeter. The duct of the submandibular gland was cannulated with a polyethylene cannula inserted distal to the intersection between the chorda lingual nerve and the duct. The amount of saliva secreted in response

to nerve stimulation was determined gravimetrically by collecting the saliva in pre-weighed tubes.

Boldyreff (1925) described the preparation of salivary fistulae in the **dog**.

For preparing a **parotid fistula**, a fine sound is introduced through the orifice of the parotid duct, which is found opposite to the largest upper molar tooth, to the depth of 6–8 cm. Around the orifice and at a distance of about 0.5 cm from it, four sutures are laid on the mucosa at equal distances one from the other. After this, a round piece of mucosae, about 1 cm in diameter around the orifice, with the sutures at the edge of this piece, is cut out with small sharp scissors. The duct is then separated from surrounding tissues about 2 cm from the orifice in the direction of its length. Then an opening is made through the cheek into the mouth (from the point half way on the vertical line from the front or the back corner of the eye to the mouth) to the base of the prepared duct. The orifice of the duct is now led outside by pulling out with the forceps. Four sutures on the piece of mucosa are made around it. The piece of mucosa is sutured carefully to the skin with knot sutures. The wound inside the mouth is closed with a continuous suture. The piece of mucosa must be covered daily with vaseline to prevent drying. Sutures must be taken out slowly, beginning 3 days after operation. For the first 10 days after operation it is necessary to produce on the dog an intensive salivary secretion, twice a day, by introducing into the mouth of the animal, dry bread or meat powder or 0.5% solution of hydrochloric acid. Saliva is collected into graduated test tubes.

In a similar way, one can produce a **fistula of the submaxillary or sublingual glands**, usually a common fistula for both glands, because their ducts have a common orifice.

Lambert et al. (1994) **cultured acinar cells** from lacrimal and submandibular glands as well as epithelial cells from rat small intestine in supplemented, serum-free media and measured the secretory components after treatment with various agents by radioimmunoassay.

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J.2 Esophagus

J.2.0.1 Tunica muscularis mucosae of esophagus *in vitro*

PURPOSE AND RATIONALE

The tunica muscularis mucosae preparation of the rat esophagus (Bieger and Triggle 1985; Ohia et al. 1992) has been recommended for evaluation of 5-HT_4 recep-

tor ligands since it possesses a homogeneous population of 5-HT_4 receptors which mediates a well defined relaxant response to 5-HT (Baxter et al. 1991, 1992; Reeves et al. 1991; Waikar et al. 1992; Eglen et al. 1993, 1995; Yang et al. 1993; Gale et al. 1994; Monge et al. 1994; Sagrada et al. 1994; Elz and Keller 1995; Hegde et al. 1995; Cohen et al. 1996, 1998; Wong and Eglen 1996; Nagakura et al. 1999; Takeda et al. 1999).

PROCEDURE

Male Sprague Dawley rats (200–300 g) are sacrificed by asphyxiation with CO_2, and 2 cm segments of intrathoracic esophagus, proximal to the diaphragm, are excised and placed in Tyrode solution of the following composition (mM): NaCl 136, KCl 2.7, $MgCl_2 \cdot 6\,H_2O$ 1.0, NaH_2PO_4 0.4, glucose 5.6, $NaHCO_3$ 11.9, $CaCl_2$ 1.8, pH 7.4. The external muscularis propria, containing the outer longitudinal and circular muscle layers of the esophagus, is carefully removed in order to isolate the inner smooth muscle tube of the tunica muscularis mucosae. The tunica muscularis mucosae is suspended in a 10 ml tissue bath containing Tyrode solution at 37 °C and aerated continuously with 95% O_2/5% CO_2. Tissues are placed under 2.5 mN tension and are left to equilibrate with Tyrode solution for 60 min (washing every 15 min) prior to starting the experiment. Responses are recorded isometrically using a Hugo Sachs Electronic (Biegestab K30) transducer coupled to a Graphtec (Linearcorder WR3 310) 4-channel chart recorder.

Preparation of tunica muscularis mucosae are contracted with carbachol (3 μM, approximate EC_{50}). Concentration-effect curves (relaxation) to 5-HT (or other agonists) are constructed in a cumulative fashion, followed by washout, with a 60-min interval between the first and second curve.

In antagonist studies, the antagonist is incubated with the tissue for 60 min following washout and the second concentration-effect curve is constructed in the presence of the antagonist. Responses are measured as decreases in isometric tension and are expressed as percentage relaxation of the carbachol-induced tone.

EVALUATION

Concentration-effect curves and agonist potencies

All agonist concentration-effect curves are fitted using an nonlinear, iterative curve fitting program according to the following relationship (Parker and Waud 1971):

$$E = E_{max}[A]^n/([A]^n + EC_{50}{}^n)$$

This relationship describes curves with a maximal response E_{max}, half-maximal response EC_{50} (both in terms of molar concentration), and a slope factor determined by the power n. [A] represents agonist concentration and E is response.

Antagonist potencies

pA_2 estimates of test compounds vs. 5-HT in the rat tunica muscularis mucosae are determined by the method of Arunlakshana and Schild (1959) and computed using Statview II (Brain Power Inc., Calabassas, CA). Concentration ratios are determined using the iterated EC_{50} values in the absence and the presence of the test compounds.

All remaining pA_2 estimates are determined by the method of Furchgott (1972) using a single concentration of agonist. The method assumes a competitive interaction and is calculated as follows:

$$pA_2 = -\log([\text{antagonist}] / [\text{concentration ratio} - 1])$$

Statistics

CL (95%) and statistical significance of the difference between samples (single comparisons; unpaired Student's t-test) are determined using Statview II.

MODIFICATIONS OF THE METHOD

De Boer et al. (1993) divided the **rat** esophagus into two parts, cervical and thoracic, each of a length of 10–15 mm. Both parts were cut longitudinally and pinned on a silicon mat with the outer, striated muscle coat up. After dissection of the striated muscle, the remaining muscularis mucosae was divided into 4 (5×2 mm, thoracic part) and 6 (5×1.5 mm, cervical part) strips. Strips from different parts showed no differences in pharmacological behavior.

Cohen et al. (1994) found 5-HT_4 receptors in rat but not guinea pig, rabbit or dog esophageal smooth muscle.

Several authors (de Boer et al. 1993, 1995; Kelly and Houston 1996; Lezama et al. 1996; Oriowo 1997, 1998) showed that β_3-adrenoceptors mediate the relaxation of the rat esophageal muscularis mucosae.

Eglen et al. (1996) studied the functional interactions between muscarinic M_2 receptors and 5-hydroxytryptamine 5-HT_4 receptors and β_3-adrenoceptors in the isolated esophageal muscularis mucosae of the rat.

Goldhill et al. (1997) investigated the 5-HT_4 receptor modulation of tachykinergic excitation of rat esophageal tunica muscularis mucosae.

The tunica muscularis mucosae of **guinea pigs** was used by various authors:

Yoshida et al. (1993) studied the effect of a gastroprokinetic agent on electrically-evoked contractions in tunica muscularis from isolated guinea pig esophagus.

Watson et al. (1995) investigated the interactions between muscarinic M_2-receptors and β-adrenoceptors in guinea-pig esophageal muscularis mucosae.

Uchida et al. (1998a,b) examined the effect of Ba^{2+} on acetylcholine- and KCl-induced contractions and characterized the endothelin-induced contraction of the guinea-pig esophageal muscularis mucosae.

Malmberg et al. (1991) studied muscle activity of isolated muscle strips from the middle pharyngeal constrictor, the inferior pharyngeal constrictor, the cricopharyngeal muscle, and the cervical esophagus from **rabbits** in organ baths in response to drugs and electrical field stimulation.

Kohjitani et al. (1993, 1996) divided the lower esophagus of rabbits into 3 regions (lower esophagus, transitional zone, lower esophageal sphincter) and studied the influence of anesthetics and peptides on contractions induced by acetylcholine or electrical field stimulation.

Percy et al. (1997) studied the pharmacological characteristics of rabbit esophageal muscularis mucosae *in vitro*.

Based on studies by Bitar and Mahklouf (1982), Biancani et al. (1987), isolated smooth muscle cells of the lower esophageal sphincter from **cats** were used by Hillemeier et al. (1996) to investigate the influence of protein kinase C on spontaneous muscle tone. Esophagus and stomach from sacrificed cats were removed and opened along the lesser curvature. The location of the squamocolumnar junction was identified, the mucosa was peeled and removed by sharp dissection under a microscope. The underlying circular muscle layer was cut into slices 0.5 mm thick with a tissue slicer. The last slices containing the myenteric plexus, longitudinal muscle and serosa were discarded. The slices of circular muscle were placed flat on a wax surface, and tissue squares were made by cutting twice with a 2-mm blade block, the second cut at right angle of the first. Isolated smooth muscle cells were obtained by enzymatic digestion with collagenase. Agonist-induced contraction of isolated muscle cells was achieved by exposing them to IP_3 and a protein kinase C agonist. The cells were fixed in acrolein at a final concentration of 1%. The length of 30 consecutive intact cells encountered at random was measured with a phase-contrast microscope and a closed-circuit video camera.

Muscle strips from *cat* lower esophageal sphincter were used by Dobreva et al. (1994), Kortezova et al. (1994), Preiksaitis and Laurier (1998).

Uy Dong Sohn et al. (1995) investigated muscle-type-specific signal transduction pathways in esophageal and lower esophageal sphincter circular smooth muscle of cats.

Tokuhara et al. (1993) studied the influence of adrenoceptor agonists on the striated muscle portion of the esophagus by use of isolated strips from **dogs**.

Saha et al. (1993) examined the effects of nitric oxide-containing compounds on **opossum** esophageal longitudinal smooth muscle *in vitro*.

La Rocca et al. (1992) examined the effects of metoclopramide in transverse muscular strips from **pigeon** esophagus.

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J.2.0.2 Esophageal sphincter *in vivo*

PURPOSE AND RATIONALE

Salapatek et al. (1992), Xue et al. (1996) studied the control of esophageal peristalsis and function of the lower esophageal sphincter in anesthetized cats.

PROCEDURE

Adult cats of either sex are fasted overnight. Anesthesia is induced with 15 mg/kg ketamine i.m. and maintained with i.v. infusion of 15 mg/kg/h. The cats can tolerate intubation while continuing to swallow spontaneously or with pharyngeal stimulation. Esophageal motility is continuously monitored by a multi-lumen catheter passed orally. The catheter has a sleeve (Dent 1976) which is positioned within the lower esophageal sphincter, with recording ports 2, 4, 6, and 8 cm above the lower esophageal sphincter and one port just below the sleeve. The recording catheter channels are continuously perfused with distilled water at 0.3 ml/min by means of a pressurized infusion pump. At the recording ports within the esophageal body, the system is able to record a pressure rise of 300 mm Hg/s. Pressures are recorded using transducers. Respiration is continuously monitored with a belt pneumograph placed around the animal's chest. All pressures are recorded on a Beckman eight-channel direct writing chart recorder with input couplers, preamplifiers and amplifiers while simultaneously taped on an eight channel FM tape recorder. Drugs are administered intravenously.

For each esophageal contraction, amplitude is measured at each esophageal level, using mean intra-esophageal pressure as baseline. Onset of contraction is determined as the point of rapid upstroke at each level, and progression of the wave along the esophagus is expressed as the lag of time or delay (in seconds) between two adjacent recording sites. Basal lower esophageal sphincter pressure is measured using intragastric pressure as reference. Maximum lower esophageal sphincter relaxation and lower esophageal sphincter after-contraction are also assessed.

EVALUATION

Statistical analysis is performed by using one-way analysis of variance and a Student's *t*-test where appropriate.

MODIFICATIONS OF THE METHOD

Greenwood et al. (1992) used a similar multi-lumen catheter assembly system in **cats** and registered additionally the electromyogram of the mylohyoid muscle.

Further studies in cats were performed by Preiksaitis et al. (1994) and Lichtenstein et al. (1994).

Using a miniature perfused sleeve/sidehole catheter, Kawahara et al. (1994) measured gastric, lower esophageal sphincter and esophageal pressure in urethane anesthetized **rats**.

Rouzade et al. (1996) monitored manometrically esophageal, lower esophageal sphincter and fundus pressure in conscious **dogs**.

Blackshaw et al. (1995), Blackshaw and Dent (1997) measured responses of the lower esophageal sphincter in urethane-anesthetized **ferrets**.

Smid et al. (1998) studied lower esophageal sphincter function in a model of oesophagitis in ferrets. Oesophagitis was induced by acid (0.15 M HCl) and 1% pepsin infusions in anesthetized ferrets. Lower esophageal sphincter strip responses were measured *in vitro* after various agents and after electrical field stimulation.

Knudsen et al. (1994) measured esophageal pressured with a four-channel, perfused catheter assembly in lightly anesthetized **opossums**.

Further studies in opossums were performed by Harrington et al. (1991), de Arruda-Henry and Uchida-Athanasio (1994).

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J.2.0.3
Permanent fistula of the esophagus in the dog

PURPOSE AND RATIONALE

Esophagostomy was made for the first time by Pavlov (1902) for the purpose of obtaining pure gastric juice from a dog. Pure gastric juice can obtained in great quantities only after shame feeding from a gastric fistula, so it is necessary that the dog shall have two fistulae: esophageal and gastric fistula. The technique to prepare a permanent gastric fistula in the dog was described by Boldyreff (1925).

PROCEDURE

It is necessary to obtain beforehand a well nourished dog with a gastric fistula. The operation must be done aseptically with the usual anesthesia. The incision on the neck is made on a median line 2–3 cm below the larynx and is 12–15 cm long. Th esophagus is found under the trachea and a little to the left. It is separated as little as possible from the surrounding tissue with a knife handle and pulled outside. The esophagus is now divided, cross-section, and the ends of the dissected esophagus are carefully sutured into the respective corners of the wound. In suturing the mucosa of the esophagus with the skin of the wound it is necessary to take into the suture the muscle layer of the esophagus also. After the ends of the esophagus are sutured into the corners of the wound, the part of the wound between them is closed. The wound should be covered with thick antiseptic ointment. After the operation it is necessary to examine the wound daily. The sutures must be taken out gradually beginning five or six days after the operation.

Oesophagotomy can be performed more simply, if the esophagus is not divided but a longitudinal incision, 12–15 cm long is made through its wall. The edges of this incision are sutured together with the edges of the wound in the neck. When the wound is healed this fistula can be temporarily closed during feeding by means of a bandage or with a special device. The dog can then eat and drink normally and the saliva is not lost through the fistula.

MODIFICATIONS OF THE METHODS

Boldyreff (1925) described also the preparation of crop fistulas in the **rooster**. A longitudinal incision 2–3 cm long is made through the skin in the middle part of the crop. Then, with the aid of a pair of surgical forceps the front wall of the crop is lifted up and an opening about 2 cm long is made in it with scissors. After this a fistular tube is introduced into the crop with the aid of a large hook and, if necessary, the opening of the crop is sutured with one suture tightly around the tube.

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J.3
Gastric function

J.3.1
Acid secretion

J.3.1.1
Acid secretion in perfused rat stomach (Gosh and Schild rat)

PURPOSE AND RATIONALE

Gosh and Schild (1958) introduced a method for the continuous recording of acid gastric secretion in the rat. The acid secretion can be stimulated by histamine, acetylcholine and gastrin. Moreover, the stimulated secretion can be inhibited by anti-ulcer drugs.

PROCEDURE
(MODIFIED AFTER GOSH AND SCHILD 1958)

Male Sprague-Dawley or Wistar rats weighing about 250 g are used. They are withheld from food, but not from water 18 h prior to the experiment. Four animals are used per dose of test drug or standard. Anesthesia is induced by i.p. injection of 5 ml/kg of 25% urethane solution. Body temperature is artificially stabilized by means of a rectal thermometer and a heating pad. The trachea is exposed and cannulated for artificial respiration. The jugular veins are then exposed and cannulated with polyethylene tubes bevelled at the tip. The abdomen is opened through a midline incision, the

pyloro-duodenal junction exposed and a double perfusion cannula (with two lumina) is introduced through a cut in the duodenum up to the cardiac part of the stomach and secured firmly by placing a ligature around the pylorus, care being taken not to include blood vessels within the ligature. Using a peristaltic pump, the stomach is perfused continuously with 0.9% NaCl solution at 37 °C. In the effluent, pH is measured with a pH-meter and continuously recorded. Gastric secretion is stimulated by continuous intravenous infusion of 100 µg/kg/h of pentagastrin or 3 mg/kg/h histamine hydrochloride or 30 µg/kg/h carbachol. As soon as acid secretion has reached a plateau, test substances or standard are injected intravenously.

EVALUATION

The maximal inhibition of acid secretion (AI_{max}) and the inhibition of acid secretion during 1 h (AI_t) are calculated.

$AI_{max} = (pH_i - pH_s) \times (pH_b - pH_s)^{-1} \times 100$

$AI_t = 100 - F_i \times F_s^{-1} \times 100$

whereby,

pH_b = pH value at basal H^+ secretion

pH_s = pH value at stimulated H^+ secretion

pH_i = highest pH value after administration of test compound

F_s = integral of pH curve over 60 min before administration of test compound

F_i = integral of pH curve over 60 min after administration of test compound.

Moreover, using various doses of the test compound and of a standard, dose-response curves can be established and activity ratios with confidence limits can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method of Gosh and Schild being modified by several authors can be used for standardization of secretagogues, like gastrin, and for evaluation of acid secretion inhibiting anti-ulcer drugs.

MODIFICATIONS OF THE METHOD

Burn et al. (1952) described the evaluation of substances which affect gastric secretion using perfusion of the stomach in anesthetized **cats**.

Lawrence and Smith (1974) described the measurement of gastric acid secretion in the **rat** by conductivity. The stomach of an anesthetized rat is continuously perfused with 2 ml/min of an isotonic (0.308 molar) glucose solution at 37 °C. The conductance of a solution depends on the total ion concentration and is therefore not specific for hydrogen ions. Since hydrogen ions have an equivalent conductance nearly 5 times greater than any other ion found in gastric juice and since they are secreted in a far greater concentration than other ions, conductivity measurements can be regarded as a relatively specific measure of hydrogen ions. Using Mullard conductivity cells (type E 791/B) and a commercially available meter (Phillips PW 9 501) simultaneous measurements in 6 rats were performed.

Gallo-Torres et al. (1979) described in detail a method for the bioassay of antisecretory activity in the conscious rat with acute gastric fistula with additional collection of the biliary and pancreatic secretion by means of a catheter in the common bile duct. The gastric secretions are collected by gravity via a cannula in the most gravity dependent site of the glandular stomach.

Larsson et al. (1983) described studies in the acutely vagotomized rat. Truncal vagotomy is performed under ether anesthesia by cutting the dorsal and ventral branches of nervus vagus just below the diaphragm. The pylorus is then ligated and a polyethylene catheter (PP 200) is inserted into the duodenum, close to the pylorus. Each animal is placed in a modified Bollman cage and is allowed to recover at least 1 h before the experiment. Gastric juice is collected by free drainage in 30 min samples.

Herling and Bickel (1986) showed that gastric acid secretion in stomach-lumen perfused rats can be stimulated *in vivo* on the subreceptor level by IBMX (phosphodiesterase inhibitor) and forskolin (non-receptor activation of the adenylate cyclase). H^+/K^+-ATPase inhibitors and H_2-antagonists show, according to their different modes of action, also a different inhibitory profile in this assay.

Hammer et al. (1992) used anesthetized female Sprague-Dawley rats weighing 200–320 g. After insertion of a tracheal cannula, a 3-mm silicon tubing is placed through the mouth and advanced to the stomach. The tubing is tied to the esophagus at the neck. A 4-mm drainage tube is inserted into the stomach through a laparatomy incision and an incision in the duodenum, and ligated in place at the pylorus. Gastric perfusate (0.9% saline at 37 °C) is collected on ice every 5 min for titration to pH 7.0.

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J.3.1.2 Isolated rat stomach

PURPOSE AND RATIONALE

The isolated whole stomach of the rat was recommended for evaluation of H_2-receptor antagonists (Bunce and Parsons 1976).

PROCEDURE

Fed immature rats of either sex weighing 38–42 g are anesthetized with sodium pentobarbitone i.p. The abdomen is opened and the esophagus ligated close to the stomach. An incision is made in the rumen of the stomach and the contents washed out with warm Krebs-Henseleit solution. A second incision is then made at the pyloric sphincter and polyethylene cannulae are inserted and tied into the stomach via these incisions. The stomach is rapidly dissected out and placed immediately into a 10 ml organ bath containing Krebs-Henseleit solution at 37 °C. The lumen of the stomach is perfused at a rate of 1 ml/min with a modified Krebs-Henseleit solution (without Na_2CO_3 and KH_2PO_4) at 37 °C. Both solutions are gassed with 95% O_2/5% CO_2. The effluent perfusate from the stomach is passed over a micro dual electrode. The changes in pH are converted to a function of hydrogen ion activity by an antilog function generator and continuously recorded on a potentiometric pen recorder. The drugs are added, in a volume not exceeding 0.5 ml, to the complete Krebs-Henseleit solution bathing the serosal surface of the stomach. After setting up the stomach preparation the basal H^+ output is allowed to stabilize, both under control conditions and in presence of the H_2-antagonist, before the secretory responses to histamine are investigated. The response to a dose of histamine is assessed by measuring the amount of acid secreted at peak response above the preceding basal level.

EVALUATION

The rate of acid secretion is expressed as [H^+] moles $\times 10^{-8}$ per min. The effect of the antagonist is assessed by measuring the potency of the agonist. An estimate of potency is firstly obtained using the agonist alone, and then a second estimate is obtained in the presence of the agonist-antagonist combination. The potency ratio is calculated according to Finney (1964).

MODIFICATIONS OF THE METHOD

A similar method was described by Szelenyi (1981) and Stanovnik et al. (1988) using the isolated stomach of the **mouse**.

Weigert et al. (1995) evaluated the effect of the opiate receptor antagonist naloxone on vagally stimulated secretion of bombesin-like immunoreactivity, somatostatin and gastrin from the isolated rat stomach which was perfused via the celiac artery with Krebs-Ringer buffer. Vagal stimulation was performed with 1 ms, 10 V and 2, 5, 0r 20 Hz, respectively.

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J.3.1.3 Chronic gastric fistula in rats

PURPOSE AND RATIONALE

A permanent gastric fistula using an especially designed Pavlov's type of cannula in the rat has been

described by Lane et al. (1957) and Komarow et al. (1960, 1963). Moreover, the preparation of chronically denervated gastric pouches in the rat has been reported by Alphin and Lin (1959).

PROCEDURE

For a **permanent gastric fistula** in rats Komarow et al. (1960, 1963) developed a stainless steel cannula of the type extensively used in Pavlov's laboratories. The cannula is 10–12 mm in length with an inner bore of 3 mm diameter and a bevelled flange at each end. The inner bore at one flange is threaded to permit insertion of a removable screw which acts as a stopper between experiments. The lumen of the cannula permits a snug fit with a No. 8 French catheter.

Male Wistar rats weighing 280–300 g are used. Under ether anesthesia, the left upper quadrant of the abdomen is shaved and the animal placed on its right side. An incision of 5–10 mm in length is made about 5 mm below and parallel to the lower left costal margin between the parasternal and mammary lines and the deep abdominal muscles are divided. The area of the rumenal wall is then drawn up through the incision. This area should be close to the greater curvature but far enough away from the glandular portion of the stomach to avoid sacrificing secretory mucosa. A small incision is made in the exposed portion of the rumen and a purse string suture is loosely placed around the margin of the opening. The unthreaded flange of the cannula is inserted into the rumen and the suture is pulled tight around the shaft of the cannula and tied. The incision is then closed by two layers of interrupted sutures. The deep abdominal muscle is closed first by a suture at each side of the cannula, using a bit of rumen to ensure a tight fit. The incision is closed with skin sutures.

During the postoperative period of 2–3 weeks, the animals are conditioned to the experimental procedure by keeping them in a restraining cage for several hours each day. Food is withdrawn 18 h prior to the experiment. The animal is placed in a restraining cage, the stopper screw removed from the cannula and the stomach gently washed twice with 2–3 ml warm saline, and 6 cm length of rubber catheter is inserted through the lumen of the cannula. Hourly collections of gastric fluid in a graduated centrifuge tube are then started. The animal model can be used for physiological studies as well as for standardization of secretagogues and for measuring the inhibition of gastric secretion by antisecretory drugs.

For preparation of **chronic denervated gastric pouches** according to Alphin and Lin (1959) male Wistar or Sprague Dawley rats weighing 250–350 g are used. Under ether anesthesia, a mid-line incision is made along the abdominal wall and the stomach is gently pulled to the outside by means of a stainless steel hook. Two small clamps are then gently applied along the stomach between the smaller and greater curvature, care being taken not to injure the blood vessels supplying the glandular and the non-glandular portions of the stomach.

An incision is made between the clamps from one end to the other end of the greater curvature with as little involvement of the mesenteries as possible. The mucosa and the muscular wall are sewed up separately. A new opening is made midway along the greater curvature of the denervated pouch into which one end of a stainless steel cannula is secured by double purse-string sutures. The stainless steel cannula is 3 cm in length with an inside diameter of 3 mm. The other end of the cannula is led through a small puncture 1 cm on the left of the midline of the abdomen. The gastric end of the cannula is firmly anchored to the body wall by silk sutures. After closing the midline incision of the body wall by silk sutures, the animal receives 150 MU procaine penicillin. The animals receive a pasted diet for some days and are kept constantly in an air-conditioned room with constant temperature during the recovery period of 2–3 weeks.

For experiments, the animals are deprived of food, but not of water prior to the test, placed in restraining cages and the gastric juice is collected in graduated centrifugation tubes. The amount of secretion into the pouch and the acidity of the fluid are measured after secretagogues, e.g. 20 mg/kg histamine s.c., alone and then two hours later after administration of potential antisecretory drugs.

EVALUATION

The effect on volume and HCl secretion at 30 min intervals after administration of the test compound is compared with the control values. The decrease is expressed as percentage of control.

MODIFICATIONS OF THE METHOD

Larsson et al. (1983) described a modified technique for the chronic fistula rat. Under methohexital anesthesia a plastic cannula is implanted in the rumen of rats close to the glandular part of the stomach wall. At the same time, a polyethylene tube (PE 50) for compound administration is inserted into the upper part of duodenum and fixed to the cannula. The duodenal tube is subcutaneously guided to the neck, where the free end is guided through the skin. One week recovery is allowed before experiments. For the experiment, the rats are placed in modified Bollman cages and 6 consecutive 1-h samples of gastric juice are collected from the gastric cannulae. After the first two collection periods, the test compounds are given intraduodenally.

Altar (1980) described design and method of implantation of a chronic gastric cannula in adult rats.

Johnson et al. (1990) described a chronic gastric cannula for feeding ethanol liquid diet to rats.

Tsukamoto et al. (1984) reported an improved method for intragastric infusion in conscious rats with simultaneous implantation of a cannula into the jugular vein for blood sampling and drug administration.

Rossowski et al. (1997) measured the inhibition of gastric secretion by adrenomedullin, amylin, calcitonin-gene related peptide and their fragments in rats equipped with chronic gastric fistulas.

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J.3.1.4 Chronic gastric fistula in dogs

PURPOSE AND RATIONALE

A simple technique for preparing chronic gastric fistulas in dogs which does not require placement of cannulae or tubes has been described by Foschi et al. (1984).

PROCEDURE

Male mongrel or Beagle dogs weighing 12–18 kg are used. After thiopental and halothane anesthesia, a round incision (2 cm) is made on the left side of the abdomen below the costal arch. The muscular fascial layers are cut across to either the peritoneum or the abdominal cavity. The stomach is grasped on the greater curvature, as high as possible, and is gently pulled through the abdominal wall. A round excision of the serous-muscular layer (0.5 cm diameter) is made, and then the mucosa is cut and sutured to the serous layer with catgut. A purse string of 3-0 silk is placed 1.5 cm around the gastrostomy hole to invert the opening and to form an antireflux flap. The stomach is also sutured to the peritoneum, the fascial layer, and the skin with catgut. After 5 days, a silver cannula has to be inserted once a week to avoid closure of the gastrostomy.

After 10–14 days, the animals are trained to lie in a cage to their left sides, supported by a 30° angle with the caudal part of the body raised to avoid the passage of gastric juice into the duodenum. For secretion studies, a short plastic tube is inserted through the fistula and gastric juice is collected for periods of 15 min. After 30 min, secretagogues (e.g., pentagastrin 6 µg/kg/min) are given as continuous infusion and samples collected every 15 min.

EVALUATION

The samples are titrated with 0.1 N NaOH to pH 7.0 by a pH meter. The results are expressed as volume (ml), pH, acid concentration (µE/ml), and output (mE/h).

CRITICAL ASSESSMENT OF THE METHOD

The technique described by Foschi et al. (1984) has the advantage of simplicity.

MODIFICATIONS OF THE METHODS

Boldyreff (1925) reported improvements and details of the technique described by Pavlov (1902).

Thomas (1941) described an improved cannula for gastric and intestinal fistulas using a removable flange being inserted separately into the stomach or intestine.

Emås (1960) reported on gastric secretory responses to repeated intravenous infusions of histamine and gastrin in non-anaesthetized and anesthetized cats with gastric fistula. A gastric cannula as well as a duodenal cannula were inserted.

Daly et al. (1980) described an apparatus for intragastric titration in the conscious dog. A titration display unit provides a record of the secretory response both as a digital printout and a bar chart display.

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J.3.1.5 Heidenhain pouch in dogs

PURPOSE AND RATIONALE

The preparation of a chronic gastric pouch, as described by Heidenhain in 1878, is one of the classic techniques in experimental surgery. This model has much contributed to the understanding of the physiology and pathology of the stomach and to modern techniques of abdominal surgery in man. The surgical technique has been described again in detail by deVito and Harkins (1957). A preparation of chronic denervated pouches in the rat has been described by Alphin and Lin (1959). Both preparations can be used as pharmacological models for testing antisecretory drugs.

PROCEDURE

Short-haired mongrel dogs or Harrier dogs weighing 15–20 kg are fasted 24 h preoperatively. Intravenous pentobarbital sodium, 30 mg/kg, provides satisfactory anesthesia. It can be supplemented, if necessary, during operation. The abdominal part is shaved with electric clippers, then with a razor. The skin is disinfected with Zephiran®-70% alcohol. Sterile drapes are applied to cover the whole surgical field. A mid-line linea alba incision from xiphoid to umbilicus provides excellent exposure and ease for closure. As the posterior sheath is divided, the large ventral fat pad present in dogs should be excised completely. A self-retaining retractor is applied and the stomach is palpated for the absence of food. Then the spleen is displaced, wrapped in warm, moist pads and laid on the ventral wall below the incision.

The stomach is pulled into the operative field. The greater curvature is held at multiple points so that the stomach is stretched out and the line of incision for the pouch is selected. The pouch should be made from the corpus of the stomach so that true parietal cell juice can be obtained. A line projected from the incisura angularis perpendicular to the proximal lesser curvature will generally fall across the junction between corpus and antrum. Appropriate division of the gastric branches of the right gastroepiploic artery at the lower end of the proposed line of transsection clears the greater curvature for 1–2 cm. The gastroepiploic artery itself should be sectioned at this site and a long rent formed on the adjacent omentum, else the omentum vessels tend to tear during subsequent manipulations.

An index finger is then inserted through this defect dorsal to the stomach to emerge higher on the greater curvature through the gastrosplenic ligament at the upper end of the proposed line of transsection. This portion of the greater curve is cleared for 1–2 cm. Von Petz clamps with their staplings are used to control bleeding and to avoid leakage of gastric content. The stomach should be kept stretched and flattened while the clamps are applied. After division between the staples, any bleeding is controlled and the cut edges of the main stomach and pouch are then oversewn with continuous sutures of black silk. The suture should be of an inverting type. Surprisingly, leakage or excessive adhesions are not a problem when serosal apposition is neglected.

The pouch so formed is about 30% of the corpus volume and provides adequate secretory volume for further studies. A cannula, made of stainless steel, 3 cm long with a bevelled flange threaded at the other end is placed in the most ventral portion of the pouch through a small incision in the anterior wall. A single purse-string of silk holds it in place. A double sheet of omentum is then wrapped about the pouch and the cannula before being pulled through the abdominal wall, about 3 cm to the left of the mid-line subcostally. It is important that the cannula be held snugly by fascia, otherwise it will readily pull out of the pouch and abdominal wall. The linea alba is closed with a continuous suture of silk and the skin with subcuticular stitches of chromic catgut. On the outside of the cannula a stainless steel jacket is screwed. For further collections, a bag is connected to the cannula by a threaded adapter, facilitating removal for daily drainage of secretions.

At the end of surgery, 300 MU penicillin and 0.5 g of streptomycin are instilled intraperitoneally. Before recovery from anesthesia the dog receives 500 ml 5% glucose in saline intravenously. The same volume is given for 3 days postoperatively together with oral fluid ad libitum. From the 4th day onward, normal food is given. Twenty-four hours secretions are collected every morning and analyzed for free HCl. A period of 7–10 days is required for full recovery from the operation. Special care has to be taken for each animal being kept separately in a suitable cage with mesh bottom.

For pharmacological studies, food is withdrawn 18 h prior to the experiment with water ad libitum. The animals are placed in Pawlow stands and a continuous i.v. infusion of either pentagastrine at a dose of 8 µg/kg/h

or histamine at a dose of 0.1 mg/kg/h is administered. Secreted fluid is collected at 15 or 30 min intervals and analyzed for free HCl. As soon as the secretion has reached a plateau, test compounds are given orally or intravenously.

EVALUATION

The effect on volume and HCl secretion at 15 or 30 min intervals after administration of the test compound is compared with the control values. Maximal inhibition is calculated as percentage change against the pre-drug value. Secretin inhibits gastric secretion volume in a dose-dependent manner. Activity ratios for unknown preparations of secretin can be calculated by 2 + 2 point assays in comparison with the international standard. Gastric secretion can be stimulated by slow continuous infusion of pentagastrin, e.g., (Gastrodiagnost®) 8 µg/kg/h.

The Heidenhain pouch technique can also be used for evaluation and standardization of **gastrin analogues**. Two or more doses of the test compound and the standard are given intravenously. Dose-response curves are established using gastric juice secretion, acid secretion, or pepsin secretion, within two 15-min collection periods as parameters. Activity ratios with confidence limits can be calculated.

For evaluation of **H_2-antagonists**, a continuous infusion of histamine is given over a period of 45 min, starting with 3×10^{-8} M/kg/h. Then, the dose of histamine is doubled and infused for the next 45 min. This procedure is repeated until secretion has reached a plateau. Secretory response curves are constructed in the presence or the absence of a H_2-antagonist at a concentration between 2×10^{-6} and 5×10^{-7} M/kg/h.

To evaluate the **type of gastric acid secretion inhibition**, e.g., cholinergic, histaminergic, or gastrinergic mechanisms, either carbachol (8 µg/kg/h), or histamine (80 µg/kg/h), or pentagastrin (8 µg/kg/h), are administered as continuous i.v. infusion until a plateau of acid secretion is reached. The experiments are performed at weekly intervals with and without the administration of various doses of test compounds. Regression lines of antisecretory effects of various doses of test drugs are constructed and used for calculation of ID_{50} values. Relative potencies versus a standard, e.g., cimetidine, can be calculated by four- or six-point assays.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described a simplified method for isolation of a portion of the stomach as compared to the original method of Heidenhain (1878).

Gastric motility can be measured by balloon manometry of the Heidenhain pouch in the conscious dog. The animals are deprived of food for 18 h before the experiment, but water is allowed ad libitum. A latex balloon, connected via a polyethylene catheter to a pressure transducer (Statham P 23 BB), is introduced through the fistula cannula into the accessory stomach. Changes in intragastric pressure are measured on a frequency measurement bridge and are recorded continuously. The number and height of the pressure waves are used as indices of gastric motor activity. Secretin inhibits gastric motility dose-dependent. After injection of gastrin or gastrin analogues, a dose-dependent increase of pressure is noted over a wide dose-range.

Jacobson et al. (1966, 1967) studied gastric secretion in relation to mucosal blood flow by an antipyrine clearance technique in conscious dogs with vagally denervated gastric fundic (Heidenhain) pouches. A vagally denervated fundic pouch was so constructed that the entire arterial blood supply was delivered by the splenic artery. A non-cannulating transducer (electromagnetic flowmeter) and a hydraulic occluder were implanted on the vessel.

The Heidenhain pouch preparation was used by Carter and Grossman (1978), Kauffman et al. (1980) to study the effect of luminal pH on acid secretion evoked by topical and parenteral stimulants and the effect of topical and intravenous 16,16-dimethyl prostaglandin E_2 on gastric bicarbonate secretion.

Baker (1979) and Roszkowski et al. (1986) developed a modified Heidenhain dog pouch preparation for collecting gastric juice exclusively from the pouch during experimental periods but allowed the pouch to be an integral part of the gastrointestinal tract during non-experimental periods. The pouch is prepared using conventional techniques but, instead of being fitted with a simple cannula through the abdominal wall, a three-way cannula is used which provides passage between the exterior orifice, the pouch and the main body of the stomach. By inserting an appropriate adapter, passage is available only to the pouch and not to the main stomach or vice versa.

The Heidenhain pouch technique in dogs has been used for preclinical evaluation of various drugs, such as:

a histamine H_2 antagonist by Uchida et al. (1993),

dual histamine H_2 and gastrin receptor antagonists by Kawanishi et al. (1997),

a 5-HT_4 receptor antagonist by Bingham et al. (1995),

an other 5-HT_4 receptor antagonist by Wardle et al. (1996),

inhibition of motilin-induced phase III contractions by pentagastrin by Yamamoto et al. (1994),

peptide YY by Zai et al. (1996),

reversible K^+-competitive inhibitors of the gastric H^+/K^+-ATPase by Parsons et al. (1995),

the antiulcer agent SWR-215 by Kataoka et al. (1997),

a selective gastrin/CCK-B receptor antagonist by Yuki et al. (1997).

Descroix-Vagne et al. (1993) used Heidenhain pouch preparations in **cats** and **rabbits** to study the effect of perfusion at pH 5.5 on acid and pepsin secretion.

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J.3.1.6 Gastrin activity

PURPOSE AND RATIONALE

Biological activity of gastrin and its analogues can be determined with a bioassay using the acid secretion as determined by pH-metry (Gosh and Schild 1955, 1958; Barrett 1966; Smith et al. 1970) or by measurement of conductivity according to Lawrence and Smith (1974).

Radioimmunoassays for gastrin have been developed (Jaffe and Walsh 1979) and are available as commercial kits.

PROCEDURE

Male Sprague-Dawley or Wistar rats weighing about 250 g are used. They are withheld from food, but not from water, 18 h prior to the experiment. Four animals are used per dose of test drug or standard. Anesthesia is induced by i.p. injection of 5 ml/kg of 25% urethane solution. Body temperature is artificially stabilized by means of a rectal thermometer and a heated pad. The trachea is exposed and cannulated. The jugular veins are then exposed and cannulated with polyethylene

tubes bevelled at the tip. The abdomen is opened through a midline incision, the pyloro-duodenal junction exposed and a catheter with two lumina is introduced through a cut in the duodenum up to the cardiac part of the stomach and secured firmly by tying a ligature around the pylorus. Care must be taken not to include blood vessels within the ligature.

Using a peristaltic pump, the stomach is perfused continuously with a phosphate-citrate buffer at 37 °C. In the effluent pH is measured with a pH-meter and continuously recorded. At the beginning, gastric secretion is stimulated by an intravenous injection of 0.5 µg/kg pentagastrin. Then, the injections are repeated in 1 h intervals with alternating doses between 0.2, 0.4, 0.8, and 1.6 µg/kg of standard or test compound. Since in this dose range linearity of the response to gastrin can be assumed a 2 + 2 point parallel assay is allowed. After each injection, pH or conductivity is measured for 45 min. The area under the curve after each dosage is evaluated by planimetry.

EVALUATION

Each of 4 animals receives two doses of standard and test compound in the order of a Latin square. The dose differences have to follow a logarithmic scale. The evaluation is performed with the 4×4 assay according to Gosh and Schild (1958).

MODIFICATIONS OF THE METHOD

Wan (1977), Chang and Lotti (1986) used CF_1 female **mice**. The whole stomach was placed in tissue baths and perfused. The effluent of the perfused stomachs was collected at 15 min intervals, and the hydrogen ion concentration was determined by titration with 0.01 M NaOH to pH 7.0, or continuously recording with a pH-meter.

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J.3.1.7 Receptor binding for gastrin

PURPOSE AND RATIONALE

Binding to gastrin receptors can be determined in isolated guinea pig gastric glands (Praissman et al. 1983; Gully et al. 1993).

PROCEDURE

The gastric glands of male guinea pigs weighing between 300 and 400 g are isolated according to Berglindh and Obrink (1976). The animals are anesthetized with 30 mg/kg Nembutal®. The abdomen is opened and the aorta is cannulated in a retrograde direction. Heparin solution (250 IU/ml) is injected forcefully through the cannula. After one min, the animal is bled through the cannula and a ligature is placed around the mesenteric vessels. The chest is opened and the thoracic aorta clamped. A warm (37 °C) phosphate buffered saline solution is pumped into the aorta, whereupon the portal vein is opened to allow a free outflow of the perfusate. By this procedure, most of the solution is forced through the gastric blood vessels. When the stomach appears totally exsanguinated, it is rapidly removed, opened along the lesser curvature and emptied. The cardial and antral regions are discarded. The corpus is rinsed several times with phosphate buffered saline solution and blotted with filter paper.

The thoroughly washed fundic mucosa from two guinea pigs is minced with fine scissors in a standard buffer consisting of 15 mM HEPES, 130 mM NaCl, 12 mM $NaHCO_3$, 3.0 mM NaH_2PO_4, 2.0 mM $MgSO_4$, 1.0 mM $CaCl_2$, 5.0 mM glucose, 4.0 mM L-glutamine, pH 7.4. The minced tissue is washed and then incubated with 0.1% collagenase in the above mentioned standard buffer containing 0.1% bovine serum albumin in a shaker bath at 37 °C. After 40 min, the glands are liberated by a series of resuspensions through a 10 ml plastic pipette, filtered through 200 µ nylon mesh (Nytex), washed and collected by centrifugation. The glands are then resuspended in 40 ml of the standard binding buffer containing 0.1% bovine serum albumin at pH 7.4. Two ml aliquots are transferred into 15 ml plastic centrifuge tubes. The amount of protein contained in fundic glands is determined according to Lowry et al. (1951).

[^{125}I]Gastrin binding is measured in the presence of 0.4 ml of gland suspension in triplicate tubes that contain 50 µl of either buffer, unlabeled gastrin (1 µM), or displacers at the desired concentration and 50 µl of [^{125}I] gastrin (70 pM final concentration). After 90 min of incubation at 37 °C, the mixture is layered over 1 ml ice-cold incubation buffer in microcentrifuge tubes and is centrifuged at 10 000 *g* for 5 min. The supernatant is discarded and the radioactivity is measured in a γ-scintillation counter.

EVALUATION

IC_{50} values and K_i constants are calculated.

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J.3.1.8 Gastrin releasing peptide/bombesin/ neuromedin

Bombesin is a tetradecapeptide originally isolated from frog skin by the group of Erspamer (Anastasi et al. 1971). Shortly before, Nakashima et al. (1970) isolated from the skin of *Rana pipiens* a peptide called ranatensin because of its contractile effects on smooth muscle. **Gastrin-releasing peptide**, which shares a C-terminus with amphibian bombesin, has been isolated from a variety of mammalian and non-mammalian species (Spindel et al. 1993). **Neuromedin B**, a decapeptide originally isolated from porcine spinal cord (Minamino et al. 1983), also shows sequence homology to bombesin and gastrin-releasing peptide. **Neuromedin N**, a six amino acid neurotensin-like peptide, shows a high affinity to brain neurotensin receptors and is rapidly inactivated by brain synaptic peptidases (Checler et al. 1990).

The peptides which belong to the bombesin family can be classified in three subgroups according to the sequence of their C-terminal tripeptide: bombesin (-His-Leu-MethNH_2), ranatensin and litorin (-His-Phe-MethNH_2), and phyllitorin (-Ser-Phe MethNH_2).

Bombesin and its homologues are known to affect a wide spectrum of biological processes (Tache et al. 1988; Parkman et al. 1994; Thomas et al. 1994; Konturek et al. 1995; Varga et al. 1994, 1995; Glad et al. 1996).

In the **gastrointestinal tract**, bombesin/gastrin-releasing peptide stimulates hormone and peptide release, stimulates gastric, pancreatic, bile and intestinal secretion, prevents gastric injury, causes smooth muscle contraction and induces epithelial growth (Dietrich et al. 1994; Kortezowa et al. 1994; Liu et al. 1995; Takehara et al. 1995; Wada et al. 1995; Weigert et al. 1995; Roberge et al. 1996; Yegen et al. 1996; Won Kyoo Cho 1997; Azay et al. 1998; Cox et al. 1998; Mercer et al. 1998; Milusheva et al. 1998; Nishino et al. 1998; Alvoro-Alonso et al. 1999; Bozkurt et al. 1999; Ladenheimer et al. 1999; Shahbazian et al. 1999).

Bombesin-related peptides inhibit **food intake** (Kirkham et al. 1994; Ladenheimer et al. 1996; Smith et al. 1997; Plamondon et al. 1998; Rushing and Gibbs 1998; Aalto et al. 1999; Edwards and Power 1999; Horstmann et al. 1999; Merali et al. 1999).

Several **central activities** of neuromedin B and gastrin-releasing peptide are reported, e.g. involvement in the hypothalamic-pituitary system (Pinski et al. 1992; Plamodon and Merali 1997; Garrido et al. 1998, 1999).

Autocrine actions of neuromedin B and gastrin-releasing peptide in small and non-small cell lung carcinomas are described (Gaudino et al. 1988; Siegfried et al. 1999).

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J.3.1.9 Bombesin receptor binding

PURPOSE AND RATIONALE

Different types receptors have been described for bombesin-like peptides (von Schrenk et al. 1989; Battey et al. 1991; Severi et al. 1991; Fathi et al. 1993). The mammalian bombesin receptor subfamily of G protein coupled receptors consists of the gastrin-releasing peptide receptor (GRP-R), also called BB2, the neuromedin B receptor (NMB-R), also called BB1, and the bombesin receptor subtype 3 (BRS-3), also called bb3 (Donohue 1999; Jian 1999). A fourth subtype, bombesin receptor subtype 4, has been isolated from a cDNA library from the brain of the frog, *Bombina orientalis* (Katsuno et al. 1999). A ligand with high affinity to all four receptors has been identified (Pradhan et al. 1998).

Moody et al. (1978) studied the binding of the radiolabeled bombesin analogue [^{125}I]bombesin to rat brain membranes. Functional GRP-preferring bombesin receptors were identified in human melanoma cells by Pansky et al. (1997). Benya et al. (1995) expressed and characterized clones of human bombesin receptors.

PROCEDURE

BALB/3T3 fibroblasts devoid of gastrin-releasing peptide receptor (GRP-R) and neuromedin B receptor (NMB-R) are selected by clonal expansion after assaying for GRP-R or NMB-R by RNase protection and binding studies (Benya et al. 1992). These BALB/3T3 cells are stably transfected using a full length human GRP-R clone (huGRP-R transfected cells) or a full length human NMB-R clone (huNMB-R transfected cells) (Corgay et al. 1991). In both cases the receptor is subcloned into a modified version of the pCD2 plasmid and transfected using calcium phosphate precipitation. Stable transfectants are isolated in the presence of 800 µg/ml aminoglycoside G-418 (GIBCO, Waltham MA), identified by binding studies, and then maintained in DMEM containing 10% fetal bovine serum and 270 µg/ml G-418. Cells are passaged every 3–5 days at confluence by splitting 1 : 4.

[^{125}I-D-Tyr^0]NMB (2 200 Ci/mmol), ^{125}I-GRP (2 200 Ci/mmol), and [^{125}I-Tyr^4]Bn (2 000 Ci/mmol) are prepared using Iodo-Gen and purified using high pressure liquid chromatography. Binding studies are performed by suspending disaggregated cells in binding buffer containing 75 pM levels of either [^{125}I-D-Tyr^0]NMB or [^{125}I-Tyr^4]Bn and 3×10^6 cells/ml for 30 min at 22 °C. Nonsaturable binding of either radiolabeled peptide is the amount of radioactivity associated with transfected cells when the incubation mixture contains either 1 µM NMB of 1 µM Bn.

The ability of various bombesin related agonists or antagonists to inhibit the binding of [^{125}I-Tyr^4]Bn to huGRP-R transfected cells and of [^{125}I-D-Tyr^0]NMB to huNMB-R transfected cells is compared.

EVALUATION

Data are expressed as the percentage of saturably bound reactivity in the absence of nonradioactive peptide. For each experiment each value is determined in duplicate, and the results are expressed as the means ±standard errors of at least 3 separate experiments.

MODIFICATIONS OF THE METHOD

Radulovic et al. (1991) studied biological effects and receptor binding affinities of pseudononapeptide bombesin/CRP receptor antagonists.

Fanger et al. (1993) identified a 63-kDa serum protein that binds somatostatin and gastrin-releasing peptide but not bombesin.

Wada et al. (1997), Ohki-Hamazaki et al. (1999) generated mice lacking the gastrin-releasing peptide receptor, the bombesin subtype-3 receptor or the neuromedin B receptor.

Akeson et al. (1997) identified four amino acids in the gastrin-releasing peptide receptor that are required for high affinity agonist binding.

Ryan et al. (1998) studied the intracellular signaling of the human bombesin orphan receptor BRS-3 by various bombesin receptor agonists and antagonists.

Chave et al. (2000) analyzed the expression of bombesin-like peptides and their receptor subtypes in normal and neoplastic colorectal tissue.

Sun et al. (2000) investigated the presence and characteristics of the functional receptors for bombesin/GRP in human prostate adenocarcinoma specimens by radio-receptor assay and the mRNA expression of the three bombesin receptor subtypes by RT-PCR.

Weber et al. (2000) determined the structure of the mouse gastrin-releasing peptide receptor gene and investigated its basal promotor activity.

Radioligands for the **GRP receptor** are:

- [^{125}I][DTyr^{6}]bombesin-6-13-methylester, [^{125}I]GRP, [^{125}I][Tyr^{4}]bombesin,

for the **neuromedin B receptor:**

- [^{125}I]BH-NMB, [^{125}I][Tyr^{4}]bombesin,

for the **bombesin receptor subtype 3:**

- [^{125}I][Tyr^{6},βAla^{11},Phe^{13},Nle^{14}]bombesin-6-14 (Alexander et al. 2000).

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J.3.1.10 Evaluation of bombesin receptor antagonists as anti-cancer drugs

PURPOSE AND RATIONALE

Bombesin and gastrin-releasing peptide affect the growth and differentiation of lung epithelium in the

fetus and the adult. Gastrin-releasing peptide is an autocrine growth factor for various small cell carcinoma cell lines. Bombesin/gastrin-releasing peptide receptors were also identified in other human cancer tissue (Halmos et al. 1995). Therefore, bombesin- and gastrin-releasing peptide-antagonists were synthesized and tested as antitumor agents (Heimbrook et al. 1991; Qin et al. 1994a, 1995; Thomas et al. 1994; Azay et al. 1996; Casanueva et al. 1996; Halmos and Schally 1997; Moody and Jensen 1998).

The procedure of Qin et al. (1994a) on the inhibitory effect of an bombesin receptor antagonist on the growth of human pancreatic cells *in vivo* and *in vitro* is described as example of various similar studies.

PROCEDURE

Cancer cell line

Cancer cells of the CFPAC-1 human pancreatic cell line, originally established from a well differentiated ductal pancreatic adenocarcinoma of a 26-year-old white male with cystic fibrosis, are routinely maintained in a monolayer culture in Costar T75 culture flasks with IMDM medium containing 10% FCS, 0.5 g/liter L-glutamine, 25 mM HEPES, 3.7 g/liter $NaHCO_3$, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B under humidified 5% CO_2 at 37 °C. The cells growing exponentially are harvested by an incubation with 0.25% trypsin-EDTA in calcium- and magnesium-free Hank's balanced salt solution for 5 min at 37 °C. For tumor cell implantation, a cell suspension is prepared in serum-free IMDM by repeatedly passing the cells through a G-22 needle; then the cells are diluted to a concentration of 5×10^6 cells/ml.

Implantation of tumors in nude mice

Male athymic BALB/c (nu/nu) 6-week-old mice are housed in a laminar airflow cabinet under pathogen-free conditions throughout the experiments. Three nude mice receive s.c. injections in the flanks with 0.2 ml of cell suspension (1×10^6 cells) and serve as tumor donors. After 4 weeks, the implanted tumors grow to a size of about 5 mm in diameter and are removed from the mice. Tumor samples are dissected free of necrotic tissue and blood vessels and are cut into small fragments of about 8 mm^3. Under ether anesthesia, two pieces of tumor fragments are implanted s.c. by trocars on both sides of the flanks for each mouse. The mice bearing the implanted tumors are randomly divided into groups with 10 mice in each group.

The nude mice with implanted tumors start to receive injections of the bombesin antagonist or vehicle 7 days after the tumor cell injection. The treatment is continued for 25 days.

Evaluation of tumor growth

During the treatment, the size of the implanted tumors is measured by calipers in each mouse at 3–4 days intervals for 25 days to construct the tumor growth curve *in vivo*. Tumor volume is calculated by the formula: Tumor volume = length × width^2 × 0.5. Tumor volume doubling time is defined as the time required for the tumors to grow from 50 mm^3 to 100 mm^3 for the control group and from 35 mm^3 to 70 mm^3 for the treatment group, respectively. The tumor growth delay time is estimated as the time difference for the treated tumors and the controls to reach a volume of 70 mm^3. At the end of the experiment, the animals are sacrificed by an overdose of ether. The tumors are removed from the animals, weighed, and immediately frozen in liquid nitrogen for measurement of DNA and protein content in tumor tissues.

Determination of DNA and protein in tumor tissue

DNA in tumor tissue is determined by the method of Labara and Paigen (1979) which is based on the enhancement of fluorescence reaction upon binding bisbenzimidazole Hoechst 33 528 to DNA in cell nuclei in a high ionic strength solution. Tumors collected in each group are pooled and homogenized in 10 times their volumes in a buffer consisting of 0.05 M NaH_2PO_4, 2.0 M NaCl, and 2 mM EDTA (pH 7.4). Hoechst 33 528 is dissolved in the same buffer at a concentration of 1 µg/ml and filtered before use. An aliquot of tumor homogenate (0.4 ml) is suspended in 4 ml of Hoechst 33 528 solution, followed by incubation in a dark room for 30 min. The reaction is measured by a fluorescence spectrophotometer at excitation and emission wavelengths of 356 and 492 nm, respectively. Calf thymus DNA type I is used as a standard.

Measurement of cell growth *in vitro*

The effects of bombesin and the bombesin antagonist on the growth of CFPAC-1 human pancreatic cells *in vitro* is evaluated by direct cell counting and [^{3}H]thymidine incorporation assay.

Direct cell counting

CFPAC-1 cells collected from 60–70% confluent cultures are used for this study and seeded to 24-well culture plates (1×10^4 cells/well). After the cells are cultured in IMDM containing 10% FCS for 48 h, the medium is replaced by IMDM supplemented with 2.5% FCS and various concentrations of bombesin, bombesin antagonist, or a combination of both. The same volume of medium but without peptides is added to control wells. Following another 24 h of incubation, the culture is terminated by aspiration of the medium from the wells and washing with PBS (0.5 ml/well).

The cells are trypsinized by a 10-min incubation with (0.5 ml/well) 0.25% Trypsin-EDTA. The detached cells are dis-persed by repeated pipetting using a G-22 needle and syringe. The number of cells is counted by an automat-ed electronic cell counter (Coulter Counter Model ZF).

[^{3}H]Thymidine incorporation assay

Single cell suspension is prepared in IMDM with 10% FCS and seeded to 24-well culture plates (1×10^4 cells/well). After 46 h of culture, the medium is changed to IMDM (0.5 ml/well) containing 2.5% FCS and various concentrations of bombesin, bombesin antagonist, or a combination of both. The same volume of medium but without peptides is added to control wells. After 24 h of culture [methyl-^{3}H]thymidine (radioactivity 25 Ci/mg) is added to each well (1μCi/well) to pulse the cells. After a 4-h incubation, the medium is removed from the wells, and the cells are fixed by Camoy's solution (1 ml/well; methanol:glacial acetic acid, 3 : 1, v/v) for 20 min. After washing 3 times with PBS, the cells in each well are dissolved with 0.5 ml of 0.3 N NaOH for 15 min at room temperature. The cell lysate is collected and mixed with 3 ml of Universal scintillation cocktail. The radioactivity is measured for 1 min by a liquid scintillation beta counter.

Receptor binding assay

Receptor binding assay is performed using intact CFPAC-1 cells in monolayer cultures. Tyr^4-bombesin is labeled with ^{125}I-Na using a Bio-Rad enzymo-bead iodination kit. Mono-^{125}I-Tyr^4-bombesin is purified by high performance liquid chromatography resulting in a specific activity of ^{125}I-Tyr^4-bombesin of about 2 000 Ci/mmol. CFPAC-1 cells are seeded to 24-well culture plates (1×10^4 cells/well) and cultured with IMDM containing 10% FCS for 48 h. The cells in subconfluent culture are washed once with serum-free IMDM supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM $MgCl_2$, 1 mM EGTA, 10 mM monothioglycerol, 0.25 mM phenylmethylsulfonyl fluoride, aprotinin 10 000 kallikrein inactivator units/liter, and 0.1% bovine serum albumin (pH 7.5), followed by an incubation for 2 h at 22 °C with the same medium containing (0.5 nM) ^{125}I-Tyr^4-bombesin in the presence or absence of various concentrations of bombesin, bombesin antagonists or structurally unrelated peptides. The binding reaction is terminated by adding 0.5 ml of ice-cold medium to each well. After washing four times with ice-cold PBS (pH 7.4) the cells in each well are dissolved with 0.5 ml of 0.3 N NaOH. The resultant cellular lysate is collected from each well for measurement of radioactivity by a gamma counter.

EVALUATION

All data are expressed as the mean ±SEM of duplicate or triplicate observations from at least 2–3 repeated experiments. Mean values between the treatment and control group are analyzed by the Student *t*-test, Mann Whitney *U*-test, or one-way analysis of variance.

Data from receptor binding assays are analyzed by a ligand-PC computerized curve-fitting program created by Munson and Rodbard as modified by McPherson (1985) to determine the types of binding sites, the dissociation constants (K_d) and the maximal binding capacity of receptors (B_{max}).

MODIFICATIONS OF THE METHOD

Similar studies, mostly by Schally's group, as with tissue derived from human pancreatic cancer were performed with nitrosamine-induced pancreatic cancers in hamsters (Szepeshazi et al. 1993, 1994, 1999), with human prostate cancer (Pinski et al. 1993a,b), with rat prostate cancer (Pinski et al. 1994a), with human gastric cancer (Halmos et al. 1994; Qin et al. 1994b), with human small-cell and non-small-cell lung carcinoma (Pinski et al. 1994b; Moody et al. 1996; Koppan et al. 1998; Kiaris et al. 1999a), with human breast cancer (Yano et al. 1994; Miyazaki et al. 1998; Kahan et al. 2000), with mouse mammary cancer (Szepeshazi et al. 1992, 1997), with human colon cancer (Radulovic et al. 1994), with human glioblastoma (Kiaris et al. 1999b), and with human renal adenocarcinoma (Jungwirth et al. 1998).

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J.3.2 Mucus secretion

J.3.2.1 Isolated gastric mucosal preparation

PURPOSE AND RATIONALE

Main and Pearce (1978) described an isolated gastric mucosal preparation from rats for studying the pharmacology of gastric secretion and the synthesis or release of endogenous substances.

PROCEDURE

Rats of either sex weighing 100–120 g or guinea pigs weighing 400–600 g are anesthetized with 60 mg/kg pentobarbitone s.c. The abdomen is opened along the midline and the stomach exteriorized. The nonglandular portion is removed and the stomach rinsed with mucosal solution, containing 136 mM NaCl, 5 mM KCl, 3.6 mM $CaCl_2 \cdot 6\,H_2O$, 1.2 mM $MgCl_2 \cdot 6\,H_2O$, and 16.7 mM glucose. The muscular layer overlying the nonantral region is separated from the mucosa by blistering. The tip of a fine needle (27 gauge) is inserted just below the muscle and mucosal solution injected between the layers. The process is repeated as many times as is necessary to blister the whole area. Fine scissors are then used to cut the muscle along the greater curvature and then parallel to the cut edge. The muscle sheet is pulled back to expose the mucosa. The stomach is then removed, opened by cutting along the greater curvature, and rinsed with mucosal solution.

Two pieces are obtained from one stomach and each is placed, mucosal surface inwards, over a 1-cm^2-opening on a polyethylene vessel (titration cup). Both preparations, one each from the ventral and dorsal surfaces, are stretched lightly over the cup and tied in place. Each tissue is placed in an organ bath, at 37 °C, containing 35 ml of serosal solution (110 mM NaCl, 5 mM KCl, 3.6 mM $CaCl_2 \cdot 6\,H_2O$, 1.2 mM $MgCl_2 \cdot 6\,H_2O$, 26 mM $NaHCO_3$, and 16.7 mM glucose) being gassed with a 95% O_2/5% CO_2 mixture. The mucosal surface is superfused by means of a peristaltic pump at a rate of 0.5 ml/min with an unbuffered solution of similar ionic composition and gassed with 100% O_2. The volume of solution on the mucosal side is kept constant by suction via the same pump using larger diameter tubing, varying between 1.6 and 2.0 ml. This small volume is used in order to follow changes in acid secretion more closely. Secretion is recorded continuously via a dual microelectrode in the mucosal solution connected with a potentiometric pen recorder. The H^+-ion concentration is noted every 15 min and expressed as apparent secretion rate. Drugs are added to the serosal solution in volumes not exceeding 1 ml. Contact times for drugs and hormones (e.g., gastrin or histamine) are between 30 and 60 min. Recovery periods before the next response are between 45 and 60 min. Responses are readily reversible on replacing the solution in the bath with fresh, warmed serosal solution.

EVALUATION

The secretory response is calculated as the increase in secretion rate at the peak of the response over the preceding basal value. Dose-response curves and time-response curves are established for histamine, gastrin, and methacholine. The effects of drugs which inhibit acid secretion are evaluated by adding them either at the peak of a response, or 15 to 60 min prior to the secretagogue.

MODIFICATIONS OF THE METHOD

Wan et al. (1974) used the fundic glandular portion of the rat stomach mounted onto a glass tube without removing the muscular layer to study the inhibition of *in vitro* stimulated gastric acid secretion by a histamine H_2-receptor antagonist.

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J.3.2.2 Primary culture of rat gastric epithelial cells

PURPOSE AND RATIONALE

Zheng et al. (1994) described an *in vitro* model for evaluation of antisecretory agents using primary cultures of rat gastric epithelial cells.

PROCEDURE

Gastric mucosal cells from 1- to 2-week-old Sprague-Dawley rats are isolated according to Terano (1982). Gastric mucosal surface is washed thoroughly with sterile cotton and Hank's balanced salt solution (HBSS) and then rinsed with HBSS before being minced into approximately 1-mm^3 pieces. The minced tissues are incubated in HBSS containing 0.1% collagenase and 0.05% hyaluronidase at 37 °C in a shaking water bath for 60 min, then pipetted several times and filtered through a sterile nylon mesh. The filtrate is washed twice with HBSS by centrifugation (200 *g* for 5 min) and resuspended in Coon's modified Ham's F-12 culture medium containing 100 µg/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycine, 15 mM HEPES, 2 µg/ml fibronectin, and 10% fetal bovine serum. Cells are seeded at a density of $1.5–2 \times 10^5$ or $1–2 \times 10^4$ cells/cm^2 directly onto either 96-well plates or 6-well plates, and maintained in a Steri-Cult incubator at 37 °C in a humidified atmosphere with 5% CO_2. The medium is changed daily. The confluent monolayers are formed after 4–5 days in 96-well plates.

Drug treatment

To measure the effects of individual drugs on the viability of gastric mucosal cells, cells are incubated with

0.02–5 mg/ml drugs in culture medium for either 2 or 48 h for the uptake of MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2*H*-tetrazolium bromide) or up to 8 days for the colony-forming efficiency assay. To measure the cytotoxicity of drugs, e.g., indomethacin, and acidified medium to gastric mucosal cells, cells are incubated in either serum-free medium containing 0.5–10 mM indomethacin for 1 h, or pH 3.5 at for 10–30 min.

To study the effect of various antacids, e.g., aluminium hydroxide or sucralfate preparations, cells are incubated for 1 h in culture medium containing these drugs. The drug suspensions are then aspirated away, followed by another hour of treatment with 3.5 mM indomethacin. Alternatively, cells are treated with drugs and 3.5 mM indomethacin concurrently for 1 h. The effects of antacids on acid-induced damage are investigated by incubating the cells with the agents for 2 h and then exposing them to pH 3.5 medium for 10 to 30 min.

MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) colorimetric assay

Cells in 96-well plates are treated with the different drugs and then incubated in 100 µl of culture medium containing 10 µl of an MTT stock solution (5 mg/ml) for 4 h at 37 °C according to Mosmann (1983). Following the incubation, 100 µl acid-isopropanol (0.04 N HCl in isopropanol) is added to the wells and incubated overnight at room temperature. The color changes are recorded at 540 nm on a microplate reader. To exclude the disturbance of precipitates in some samples, the samples are centrifuged and only the supernatants are read. For each experiment, a standard curve is generated by measuring the relationship of absorbance to a series of viable cell numbers.

Neutral red uptake assay

Neutral red is prepared as a 1% stock solution in distilled water and diluted to 0.035% in HBSS immediately before each experiment. The cells are treated with the drugs and then stained with 0.1 ml of 0.035% neutral red for 30 min (Parish and Müllbacher 1983). The stain is discarded and the cells are washed twice in HBSS before the addition of 200 µl/well of acidified alcohol solution (50%, v/v, ethanol/water, containing 0.5% acetic acid). After a 2 h incubation period at room temperature, the color changes are measured in a microplate reader.

Colony-forming efficiency assay

The cells are seeded into 6-well plates and incubated in culture medium containing 0.02 to 5 mg/ml of individual drugs for 8 days. Cells are then fixed in 10% formalin and stained with 1% aqueous crystal violet (Sundqvist et al. 1989). Colonies formed on each well are counted (crystal violet stains cell nuclei) and compared with those formed on drug-free wells.

EVALUATION

Data are expressed as mean ± standard error. A one-way analysis of variance followed by Scheffe's post hoc test is used to test the significance between control and drug-treated samples. Differences are considered significant at $P < 0.05$.

MODIFICATIONS OF THE METHOD

Buchan et al. (1993) used cultured human antral epithelial cells enriched for D cells to study the effect of cholecystokinin and secretin on somatostatin release.

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J.3.3 Gastric motility

J.3.3.1 Measurement of intragastric pressure in rats

PURPOSE AND RATIONALE

Gastric motor activity can be measured by recording intragastric pressure in anesthetized rats (Holzer 1992).

PROCEDURE

Sprague-Dawley rats of either sex weighing 220–240 g are deprived of food for 20 h prior experimentation but are allowed free access to tap water. After induction of anesthesia by i.p. injection of phenobarbital sodium (0.92 mmol/kg), the trachea is cannulated to facilitate spontaneous breathing. The left carotid artery is cannulated and connected to a pressure transducer to monitor mean arterial blood pressure. The left jugular vein is cannulated for i.v. injection of drugs and for continuous infusion of physiological saline at a rate of 0.6 ml/h to avoid dehydration of the animals. Intragastric pressure is measured by a catheter (outer

diameter 1.9 mm) passed down to the stomach via the esophagus. The position of the catheter tip in the corpus region is verified at the end of each experiment. The catheter has two side holes in its tip segment and is continuously perfused with physiological saline at a rate of 0.6 ml/h. To record intragastric pressure, the catheter is connected to a pressure transducer.

EVALUATION

The gastric motor effect is quantitated by calculation of the area under the curve versus baseline intragastric pressure, measured immediately before injection of the stimulant (e.g., neurokinin A). To test inhibitors, the area under the curve after the stimulant is set as 100% and the effect of application of the inhibitor with the stimulant is calculated as percentage.

MODIFICATIONS OF THE METHOD

Lotti et al. (1986) described a simple mouse assay for the *in vivo* evaluation of cholecystokinin antagonists which is based on visual determination of the gastric emptying of a charcoal meal.

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J.3.3.2 Isolated smooth muscle preparation of guinea pig stomach

PURPOSE AND RATIONALE

Boyle et al. (1993) described a novel smooth muscle preparation from the guinea pig stomach for characterization of CCK receptors by use of selective antagonists.

PROCEDURE

Adult male guinea pigs weighing 330–400 g are sacrificed and the stomach rapidly removed. The fundus is discarded and the stomach is opened along the greater curvature, pinned on a Petri-dish with the mucosa pointing up. The mucosal and submucosal layers are removed by dissection to reveal the underlying smooth muscle layer. Strips of circular muscle (2×25 mm) from the corpus region of the stomach are obtained by cutting inwards, following the striations of the lesser curvature. Muscle strips are mounted in siliconized 3 ml organ baths containing Krebs-Henseleit solution. The buffer is modified to include 5 μM indomethacin. The solution is maintained at 37 °C and continuously gassed with a mixture of 95% O_2/5% CO_2. Isometric contractile responses are measured with force-displacement transducers (e.g., Grass FT.03) and recorded on a polygraph (e.g., Mark VII Graphtec Linearcorder).

Tissues are placed under 1 g tension and allowed to equilibrate for 30 min after which time they are contracted with a submaximal dose of carbamylcholine (10 nM). Using a 12 min dose cycle, concentration-response curves are established for agonists, e.g., gastrin, pentagastrin, CCK and CCK-analogues. For studies with antagonists, the tissues are exposed to antagonists for 15 min before re-exposure to agonists. The addition of 5 μM indomethacin removes the spontaneous activity due to the inherent myogenic tone existing in the tissue, but leaves the responses to the agonists unaffected.

EVALUATION

Contractile responses to exogenously applied agonists are expressed as absolute changes in tension and are transformed as a percentage of the maximal response achieved for that agonist in order to obtain potency values. EC_{50} values are obtained graphically for individual concentration-response curves. Responses to agonists in the presence of antagonists are expressed as a percentage of the control maximum response obtained in the same tissue preparation. Agonist concentration-response curves in the absence and presence of increasing concentrations of antagonists are obtained. The method of Arunlakshana and Schild (1959) is used to provide Schild plots of the data, and to obtain affinity constants for the antagonists.

MODIFICATIONS OF THE METHOD

Riazi-Farzad et al. (1996) described an improved preparation of the **isolated rat stomach fundus strip** based on the finding that the majority of the contractile response to 5-HT and carbachol was present in the left ventral longitudinal quartile of the tissue.

Van Nueten et al. (1978), Reyntjens et al. (1984), Schuurkes et al. (1985), Kishibayashi and Karasawa (1998) used an **isolated gastroduodenal preparation of the guinea pig**. After ligation of the esophagus, the stomach was filled with 20 ml saline and suspended in 200 ml of oxygenated Krebs-Henseleit solution, maintained at 37 °C. The duodenum was cannulated and connected with an ultrasonic transit time transducer to record changes in intraluminal volume and with a bottle of saline to ensure constant hydrostatic pressure of 6 cm H_2O. Spontaneous phasic activity was always present on the stomach and recorded as rhythmic changes in gastric volume. Gastric peristaltic waves either stopped at the pylorus or were propagated to the duodenum. Antroduodenal coordination was quantified as the relative number of antral waves that were propagated to

the duodenum. After 30 min stabilization, drugs were added in varying concentrations and the effects followed for 30 min. Three parameters were determined: (1) contractile amplitude, expressed as milliliters of expelled volume; (2) frequency, measured as number of contractions per minute; (3) percentage of antroduodenal coordination.

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J.3.4 Absorption

J.3.4.1 Measurement of gastric absorption of drugs in rats

PURPOSE AND RATIONALE

Although the stomach is not the prime absorptive site of drugs, the absorption of some drugs from the gastric mucosa has been established (Doluisio et al. 1969; Welling 1977). Worland et al. (1983) described an *in situ* gastric pouch technique for direct measurement of the gastric absorption of drugs in the rat.

PROCEDURE

Male Wistar rats weighing 220–300 are anesthetized by i.p. injection of 50 mg/kg sodium pentobarbital. A cannula is placed in the jugular vein for administration of heparinized saline (3 000 IU/kg) and whole blood replacement during the experiment. The replacement blood is collected from heparinized donor rats immediately prior to the experiment. Another cannula is placed into the carotid artery of the experimental animal for the collection of systemic blood samples. A midline incision is made in the abdominal wall. Double ligatures are placed on the superior epigastric vessels and transverse incisions are made between the ligatures. The branches of the right gastroepiploic veins and arteries are then ligated using surgical silk and the gastrohepatic ligament between the stomach and the posterior surface of the left hepatic lobe and the caudal hepatic lobe is separated. The gastrosplenic mesentery is also severed. A strip of gauze is employed to keep the liver lobes out of the surgical field. Using appropriately sized surgical silk, double ties are placed around the short gastric vessels and the esophagus, which are then severed between the ties. The pylorus and pyloric vessels are then ligated and an incision is made in the forestomach to allow the removal of gastric contents.

The pouch is then rinsed with warm saline until clear and the remaining fluid is removed using an adsorbent tissue. A Luer adapter modified from a three-way tap is tied into the incision to enable drug administration via a syringe. The gastric pouch is then transposed to the right of the animal exposing the left gastric vein. The vein is cannulated above the junction with the lienal (splenic) vein using a 21-gauge needle connected to a 15-cm length of polyethylene tubing (i.d. 0.75 mm, o.d. 1.45 mm). A small Oxford clamp with foam rubber insets is employed to prevent dislocation of the cannula during the changing of the sample vials. The drug is administered into the stomach and blood draining from the gastric pouch is collected over timed intervals. To determine the volume (ml) of blood collected, the venous effluent is weighed and hematocrit (HCT) readings are taken for each sample and converted to units using the formula:

$$\text{Volume (ml)} = \frac{\text{mass blood collected (g)}}{\text{HCT} \times \text{blood cell density} + (1 - \text{HCT}) \times \text{plasma density}}$$

Rat blood cell and plasma density measurements are obtained from five determinations from four rats, blood samples being separated at 3000 *g* for 10 min. Samples from the gastric pouch are kept on ice until the plasma can be separated by centrifugation at 3000 *g* for 10 min and the plasma frozen until assay.

Blood replacement is delivered at a rate of 0.7 ml/min from a gently oscillating reservoir using a peristaltic pump.

The compound to be tested is administered in a volume of 0.5 ml to the gastric pouch. Plasma levels are determined with a method specific for the compound under investigation. Plasma samples are collected at 4 min intervals over a period of 30–60 min. At the end of the experiment, the fluid in the gastric pouch is collected for determination of the dose remaining in the stomach.

EVALUATION

Mean values ± standard deviation of plasma concentration are calculated from 4–6 experiments and plotted versus time to demonstrate the absorption profile.

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J.3.5 Antacid activity

J.3.5.1 Evaluation of antacids

PURPOSE AND RATIONALE

Antacids have been used for the treatment of gastroduodenal ulcerations since a long time (Konturek 1993). The main action of antacids is to reduce the acidity of the gastric content through neutralization and increasing intragastric pH. Since pepsin is not active at higher pH levels, antacids reduce peptic activity (Goldberg et al. 1968) and may also adsorb pepsin (Sepelyak et al. 1984). Binding of bile salts (Clain et al. 1977) by antacids may also have a beneficial influence on peptic ulcer disease. Most antacids contain magnesium and aluminum hydroxide, in some cases also calcium carbonate and sodium bicarbonate. The acid-neutralizing potency can be measured *in vitro*.

PROCEDURE

0.1 M HCl is added to the antacid to be tested. The acid neutralizing capacity is defined as the amount of 0.1 M HCl that can be added to a liquid antacid without reducing the pH of the mixture below pH 3.0. The determination of the acid neutralizing capacity of antacid tablets is performed *in vitro* by stirring a mixture of crushed tablets and water. The time at which all antacid is consumed is much longer for antacid in tablet as compared with the same amounts of antacid in liquid form. Magnesium hydroxide is very insoluble in water, but is readily soluble in hydrochloric acid. In combined preparations, the magnesium hydroxide reacts first to produce an almost immediate neutralizing effect and an increase of the pH within a few minutes. Aluminum oxide, on the other hand, has a weaker antacid activity, reacting more slow with acid (Fordtran et al. 1973; Richardson et al. 1988).

EVALUATION

In vitro titration curves of 0.1 M HCl with 5 ml liquid antacid or one tablet are measured over 3 h and compared with the standard.

CRITICAL ASSESSMENT OF THE METHOD

Acid neutralizing potency may be not the only factor which contributes to the therapeutic effect of antacids. Damage and protection in the stomach are essentially represented by acid secreted by the parietal cells and by bicarbonate released by the surface epithelial cells and mucous neck cells. Among various neurohumoral factors most important in the bicarbonate-secretion appear the prostaglandins, mainly of the E series. Mucosal bicarbonate secretion may be stimulated through the activation of prostaglandins by aluminum hydroxide containing antacids. Aluminum containing antacids were found to protect against mucosal damage due to topical irritants and against stress or aspirin induced lesions. Gastroprotection of aluminum-containing antacids has been attributed to the biological activity of nitric oxide interacting with mucosal prostanoids on the mucosal microcirculation. Therefore, these antacids were found to be active in various experimental ulcer models and to exert cytoprotective activity against ethanol induced gastric injury in rats (Szylenyi et al. 1983; Domschke et al. 1986; Hollander et al. 1986; Konturek et al. 1989, 1992; DiJoseph et al. 1989; Vergin and Kori-Lindner 1990). Konturek (1993) reported that aluminum hydroxide containing antacids protect growth factors, involved in the healing of ulcers, against acid degradation.

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J.3.6 Inhibition of HCl secretion

J.3.6.1 Anticholinergic activity

J.3.6.1.1 General considerations

Gastric motility and tonus, as well as gastric secretion are stimulated by cholinergic impulses. Anticholinergic compounds, such as the *Belladonna* alkaloid atropine, were the first drugs used in treatment of gastric ulcers. The doses that are necessary to reduce acid secretion also decrease mucus secretion and cause side effects, such as dry mouth, increase in heart rate and ocular disturbances. Inhibitors of specific muscarinic receptors responsible for gastric acid secretion were found. Receptors for acetylcholine which play a major role in central and peripheral transmission have been studied extensively (Karlin et al. 1976; Hulme et al. 1990; Jones et al. 1992; Kebabian and Neumeyer 1994).

J.3.6.1.2 Acetylcholine receptor binding

PURPOSE AND RATIONALE

The search of specific muscarinic antagonists for inhibition of gastric acid secretion lead to the discovery of pirenzepine, a tricyclic compound, originally tested as psychotropic agent. This compound has a greater gastrointestinal selectivity than other muscarinic antagonists (Carmine and Brogden 1985; Longdong 1986). The activity of pirenzepine has been localized to M_1 receptors.

This, and the involvement of acetylcholine in many physiological processes, has stimulated the research on the various types of muscarinic receptors. At present, 5 types have been described but further subdivisions can be envisaged (Hulme et al. 1990; Jones et al. 1992; Kebabian and Neumeyer 1994; Alexander et al. 2001). The subtypes of muscarinic receptors have been characterized by the use of organs with a predominant subtype receptor population, e.g., rabbit vas deferens stimulated with electric impulses for M_1, electrically stimulated left atria from guinea pigs for M_2, and longitudinal smooth muscle preparations of guinea pig ileum or salivary gland of the rat for M_3 (Doods et al. 1987; Lambrecht et al. 1993), or by the use of selective antagonists (Pitschner et al. 1989; Richards 1990; Svensson et al. 1992,) or agonists (Lambrecht et al. 1993). Further characterization has been achieved by studies of the transduction mechanisms (Brown and Brown 1984; Brown et al. 1985; Parekh and Brading 1992) and by voltage clamp techniques (Bernheim et al. 1992). The genes for the muscarinic acetylcholine receptor subtypes have been cloned and expressed in Chinese ovary hamster cells (Buckley et al. 1989; Dörje et al. 1991) or in fibroblasts (Kashihara et al. 1992).

Many organs, e.g., rat brain (Luthin and Wolfe 1984; El-Fakahani et al. 1986) have been used for studies on acetylcholine receptor subtypes. Only the method using Chinese ovary hamster cells is presented here.

PROCEDURE

Preparation of plasmid DNA

The coding sequences of the m1, m2, m3, m4, and m5 receptors are derived from a human genom library. The cDNAs are inserted into the Okayama/Berg pCD or pCD-PS expression vector (Bonner et al. 1987, 1988; Buckley et al. 1989). Plasmid DNA is isolated by two sequential density gradient centrifugations through CsCl (Maniatis et al. 1982).

Cell culture

Chinese hamster ovary cells are incubated at 37 °C in a humidified atmosphere (5% CO_2) as a monolayer cul-

ture in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml each of penicillin G and streptomycin, and 4 mM glutamine.

Transfection procedures

Cells are transfected using a modified calcium phosphate procedure (Chen and Okayama 1987) involving the use of cotransfected pcDneo as a selectable marker. Selection with the neomycin analog G 418 (600 µg/ml; Gibco NY) is started 72 h after transfection and cont-in-ued for 2–3 weeks. Media are changed every 3 days. Clonal cell lines are obtained by single cell cloning and assayed for [^{3}H]NMS (N-methylscopolamine hydrochloride) binding capacity.

Membrane preparation

Cells are grown to about 80% confluency, scraped into ice-cold binding buffer and homogenized for 30 s using a Brinkman homogenizer (setting 5). Membranes are pelleted at 16 000 *g* for 15 min and re-homogenized. Membrane protein is determined using a Bio-Rad protein assay dye reagent. Membranes are stored frozen at –80 °C before use.

Radioligand binding studies

All membranes, drugs, and radioligands are made up in binding buffer consisting of 25 mM sodium phosphate (pH 7.4) containing 5 mM magnesium chloride. The assays are performed in 1 ml total volume. Final membrane concentrations are: m1 6 µg/ml; m2 10 µg/ml; m3 5 µg/ml; m4 3 µg/ml; and m5 4 µg/ml. In [^{3}H]NMS saturation experiments, 8–10 different concentrations of the radioligand (2–1 400 pM) are employed. For displacement experiments, [^{3}H]NMS in a concentration of 150 pM and 10 different concentrations of the displacer are used. Specific binding is defined as the difference in [^{3}H]NMS binding in the absence and presence of 1 µM atropine. Alternatively [^{3}H]pirenzepine is used. Incubations are carried out at 22 °C for 3 h. Assays are terminated by filtration through a Brandell cell harvester onto Whatman GF/C filters. Membranes are washed three times with 5 ml of ice-cold binding buffer before being dried. They are transferred to 10 ml of scintillant (New England Nuclear Aquasol) and counted in a LKB β-counter.

EVALUATION

Data from direct binding experiments are fitted to the equation:

$$\alpha = (B_{max}\, x^n / k) / (1 + x^n / k)$$

to derive the Hill coefficient n and to:

$$\alpha = (B_{max}\, x / K_D) / (1 + x / K_D)$$

to obtain the dissociation constant K_D and the total number of binding sites B_{max} (α = [^{3}H]NMS specifically bound; x = [^{3}H]NMS concentration).

Data from displacement experiments are fitted to the equation:

$$\%\ [^3\text{H}]\text{NMS bound} = 100 - [100\, x^n / k / (1 + x^n / k)]$$

to obtain the Hill number n and to:

$$\%\ [^3\text{H}]\text{NMS bound} = 100 - [100 \times IC_{50} / (1 + x / IC_{50})]$$

to derive the IC_{50} value (x = concentration of the cold inhibitor).

K_i values are calculated by the method of Cheng and Prussoff (1973):

$$K_i = IC_{50} / (1 + L / K_D)$$

where L is the concentration of the radioligand, IC_{50} is the concentration causing 50% inhibition of the specific radioligand binding and K_D the dissociation constant of the radioligand receptor complex. Data are analyzed by a non-linear least-squares curve fitting procedure.

Results are expressed as mean values ±SEM of n experiments. Statistical significance is assessed using Student's *t*-test or Scheffé's method. $P < 0.05$ is accepted as being significant.

MODIFICATIONS OF THE METHOD

The selectivity towards muscarinic receptor subtypes can be tested by radioligand binding assays using either selective ligands or tissues possessing only one receptor subtype (Giachetti et al. 1986; Pitschner et al. 1989; Bickel et al. 1990). M_1-receptors from bovine cortex which has also M_2-and M_3-receptors are tested with the M_1-selective radioligand ^{3}H-pirenzepine. M_2-receptor from porcine heart possessing only this receptor type can be tested with the unselective ligand ^{3}H-N-methylscopolamine. M_3-receptors from rat submaxillary gland are likewise labelled with the unselective ligand ^{3}H-N-methylscopolamine, because this subtype is present predominantly in this tissue.

Measurement of the contractions of rabbit vas deferens after electrical stimulation was used to study the effects of prejunctional M_1 heteroreceptors and postjunctional M_2 receptors (Eltze et al. 1988; Dörje et al. 1991), of guinea pig atria for M_2-receptors and of guinea-pig ileum for M_3-receptors (Lambrecht et al. 1989, 1995). Cardiac muscarinic $M_{2\alpha}$ receptors have been discussed (Wess et al. 1988).

Coexistence of M_2 and M_3 subtypes of muscarinic receptors in canine colonic circular smooth muscle was reported by Zhang et al. (1991).

Investigations on the nature of muscarinic receptors present on parietal cell membranes using binding studies and polymerase chain reaction amplification or parietal cell messenger RNA revealed the existence of only a M_3 receptor responsible for acid secretion (Kajimura et al. 1992).

The phenotypes of knockout mice or the responses lost in these animals were used to characterize the properties of the subtypes of muscarinic receptors (Birdsall et al. 2001).

Owicki et al. (1990, 1992), McConnell et al. (1991, 1992) used a special apparatus, the 'cytosensor microphysiometer' which measures the rate of proton excretion from cultured cells. Chinese hamster ovary cells were transfected with the m_1 muscarinic acetylcholine receptor. Sequential addition of increasing doses of carbachol every 2.5 min induced an increasing acidification allowing the determination of an EC_{50} value. The effect was antagonized by atropine.

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J.3.6.2 H_2-antagonism

J.3.6.2.1 General considerations

Three classes of histamine receptors with subtypes for the H_3 receptor have been identified by selective antagonists (Haaksma et al. 1990; Hill 1990; West et al. 1990; Clapham and Kilpatrick 1992). H_2-antagonists inhibit competitively the interaction of histamine with H_2-receptors responsible for acid secretion in the stomach. Although H_2-receptors are present in many tissues, including vascular and bronchial smooth muscle and the right atrium, H_2-antagonists interfere remarkably little with physiological functions other than gastric secretion.

The H_2 receptor was reported to be spontaneously active in transfected CHO cells (Smit et al. 1996). Based on this concept, the H_2 antagonists were reclassified; cimetidine, ranitidine and famotidine are in fact inverse agonists, whereas burimamide acts in this model system as a neutral agonist.

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J.3.6.2.2 Histamine H_2-receptor binding

PURPOSE AND RATIONALE

Histamine H_2-receptor binding can be determined using homogenates from guinea pig cerebral cortex and ^{3}H-tiotidine as labeled ligand (Gajtkowski et al. 1983; Norris et al. 1984; Hill 1990). Using the polymerase chain reaction, the gene encoding the histamine H_2 receptor has been cloned (Gantz et al. 1991).

PROCEDURE

Preparation of membranes

Guinea pigs of either sex weighing 400–600 g are sacrificed by exsanguination and the brains rapidly removed. The cerebral cortex is dissected away from the rest of the brain and homogenized in 50 mM sodium-potassium buffer, pH 7.4, using a Potter homogenizer. The homogenate is then centrifuged at 50 000 *g* for 10 min at 4 °C. The resulting pellet is washed three times by being resuspended in phosphate buffer followed by recentrifugation. The pellet is finally resuspended in phosphate buffer, pH 7.4, at a protein concentration of 5 mg/ml.

Assay

An aliquot of 100 µl of the homogenate is incubated with 2 nM ^{3}H-tiotidine and varying concentrations of competing test substance, in triplicate, in a total volume of 250 µl, for 30 min at room temperature. The reaction is stopped by the addition of 2 ml of ice-cold phosphate buffer and immediately filtered under reduced pressure through Whatman GF/B glass-fiber filters, followed by 3 times 3 ml washes with room temperature buffer.

Radioactivity is determined by allowing the filters to remain for at least 18 h in NE 260 scintillator (Nuclear Enterprise), followed by liquid scintillation counting.

EVALUATION

K_i values (µM) are calculated for displacement of specific H_2-binding from the relationship

$$K_i = IC_{50} / (1 + [L]\, K_d^{-1})$$

where IC_{50} is the concentration of the drug required for 50% inhibition of specific binding, [L] is the concentration of ^{3}H-tiotidine in the assay and K_d is the dissociation constant for ^{3}H-tiotidine.

Data can be analyzed using a computer program as described by McPherson (1985).

MODIFICATIONS OF THE METHOD

Hirschfeld et al. (1992) performed photoaffinity labeling studies of the H_2 receptor using the radioactive probes [^{125}I]iodoaminopotentidine and its photolabile azido analogue [^{125}I]iodoazidopotentidine.

Martinez-Mir et al. (1990) studied the distribution of histamine H_1, H_2, and H_3 receptors in postmortem human and rhesus monkey brain by receptor autoradiography using [^{125}I]iodobolpyramine, [^{125}I]iodoaminopotentine, and [^{3}H](R)α-methylhistamine as ligands to label H_1, H_2, and H_3 receptors, respectively.

Traiffort et al. (1992) used [^{125}I]iodoaminopotentidine for pharmacological characterization and autoradiographic localization of histamine H_2 receptors in human brain.

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J.3.6.2.3 H_2-antagonism in isolated guinea pig right atria

PURPOSE AND RATIONALE

H_2-antagonism can be determined in isolated guinea pig right atria which contain predominantly H_2-receptors. Compounds that inhibit the positive chronotropic effect mediated by histamine H_2-receptors in the isolated right guinea pig atrium (Reinhardt et al. 1974) can be classified as specific histamine H_2-antagonists. The test can be used as screening method for H_2-antagonists.

PROCEDURE

Male guinea pigs, e.g., Hartley strain, are sacrificed by exsanguination. The right atria are dissected and suspended at 0.7 g tension in a 25 ml organ bath with Tyrode's solution bubbled with carbogen (5% CO_2/95% O_2) at 38 °C. After a stabilization period of 30 min, the contractions are recorded on a polygraph with a force-displacement transducer through a strain gauge. Cumulative concentration-response curves are obtained after sequential additions of histamine (10^{-7} to 10^{-4} M) in the absence of test drugs and the equilibration of test drugs or the standard (cimetidine).

EVALUATION

pA_2 values and relative potencies are calculated by a Schild plot (Arunlakshana and Schild 1959).

MODIFICATIONS OF THE METHOD

Hattori et al. (1990) studied the inotropic, electrophysiological and biochemical responses to histamine in rabbit papillary muscles and found evidence for coexistence of H_1- and H_2-receptors. Histamine increases force of contraction and decreases action potential duration in rabbit papillary muscles. H_2-antagonists antagonize the histamine-induced decrease in action potential duration, however, are less effective in antagonizing the increase in force of contraction produced by histamine. The positive inotropic response to histamine is abolished by sequential addition of a H_1-antagonist.

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J.3.6.2.4 H_2-antagonism in isolated rat uterus

PURPOSE AND RATIONALE

Histamine inhibits spontaneous and electrically stimulated contractions of rat uterus horns. This effect can be antagonized by H_2-, but not by H_1-antagonists.

PROCEDURE

Adult, virgin Wistar rats weighing 180–200 g are used. By microscopic examination of vaginal smears, the stage of the estrus cycle is determined. Only animals in natural proestrus or metestrus are chosen. After sacrifice, the two uterus horns are removed and placed in modified de Jalon solution at room temperature and cleaned of mesenteric fat and connective tissue. Paired preparations from the same animal are used in parallel experiments. Two segments, about 2 cm in length, are taken from the ovarian end of the uterus horns and mounted separately on Perspex organ holders between two steel electrodes arranged at the end of the muscle. The organs are immediately superfused with modified de Jalon solution at 35 °C at a rate of 2–3 ml/min being continuously gassed with air. Basal muscle tension is maintained at 0.5 g. The contractions of the preparations are recorded isometrically with a force-displacement transducer linked to a multipen recorder. The intrinsic rhythm of spontaneous and regular contractions appears within 5–10 min after mounting the tissues in their holders.

Organs showing a frequency of only one contraction every two min or less are electrically stimulated with square wave impulses of 2 ms duration for 1 s every 1 min at 10 V and 80 Hz with a suitable stimulator. The organs are linked via a four-way stopcock to a set of reservoirs of modified de Jalon solution containing different concentrations of agonist and test substance. The preparations are superfused for 20–30 min with nutrient solution until mechanical activity of the uterus horns is stabilized in frequency and contraction height before addition of drugs.

The time interval between application of drugs is 15–20 min, the time of contact of the drugs with the uterus segments is about 4–5 min, being interrupted as soon as the contractions of the muscles reach a minimum. Logarithmic dose-response curves for histamine are constructed from mean effects of single doses, taking the average amplitude of 5 contractions immediately preceding the addition of histamine as 100% control activity. In experiments involving antagonists, a standard dose of histamine is used giving a response within the linear region of the log dose-response curve and is set as 100%. Reduction of histamine-induced inhibition is studied by superfusing increasing concentrations of antagonists in addition to the standard dose of histamine.

EVALUATION

Results are expressed in percentage of the reversal of initial depression of contraction height after histamine and plotted against –log mol/l concentration of the antagonist. pA_2 values are calculated according to Ariëns and van Rossum (1957).

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J.3.6.2.5 Activity at histamine H_1- and H_2-receptors *in vivo*

PURPOSE AND RATIONALE

Owen and Pipkin (1985) described a technique that allows simultaneous and quantitative assay of the action of agonists and antagonists at histamine H_1- and H_2-receptors in anesthetized guinea pigs. The principle of the technique is based on H_1-receptor caused bronchoconstriction and H_2-receptor caused tachycardia.

PROCEDURE

Guinea pigs of either sex weighing about 500 g are anesthetized with 90 m/kg sodium pentobarbitone i.p. The trachea is cannulated to permit artificial respiration. Blood pressure is measured from a catheter tied into one carotid artery. Catheters are tied into one jugular and one femoral vein for the administration of drugs.

Airways resistance is measured using a modification of the Konzett-Rössler technique. By means of a small animal respiration pump, the lungs are inflated at a rate of 40 breaths/min. The inflow arm of the circuit includes a side-arm, the outlet of which is placed below 12 cm of H_2O. The volume of air used in the study has to be that which fills the lungs at a pressure of 12 cm H_2O, selected by adjusting the volume until no air escapes through the H_2O trap each time the lungs are filled. The side-arm is then clamped, and the animal is respired with the selected volume for the duration of the study. Airways resistance to inflow is measured using a pressure transducer connected to a second arm of the inflow circuit. Resistance is proportional to the maximum pressure required to inflate the lungs. Inflow pressure is registered on an electronic recorder.

Heart rate is measured from the blood pressure pulse using an instantaneous rate meter and is registered on an electronic recorder.

Intravenous injection of histamine causes simultaneously bronchoconstriction and tachycardia. The threshold dose needed to cause tachycardia may be less than that for bronchoconstriction, but both responses will be apparent over the dose range 1×10^{-8} to 5×10^{-7} mol/kg.

EVALUATION

Agonists

Dose-response curves for agonists compared to the dose-response curve for histamine for both parameters, bronchoconstriction and tachycardia, allow the calculation of potency ratios. Specific H_1-receptor agonists are more potent to cause bronchoconstriction than tachycardia, whereas H_2-agonists provoke tachycardia but are less active or inactive causing bronchoconstriction.

Antagonists

To evaluate antagonists, various doses of the test compound are injected i.v. and dose-response curves of histamine are established for both parameters to be compared with the dose-response curve of histamine without pretreatment. From the ratios of shift to the right dose-response curves for both parameters can be established. These are similar for mixed antagonists, but a definitively higher potency is shown for H_1-antagonists in bronchoconstriction and a higher potency for H_2-antagonists in tachycardia.

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J.3.6.2.6 Inhibition of histamine stimulated adenylate cyclase from gastric mucosa

PURPOSE AND RATIONALE

The *in vitro* potencies of H_2-antagonists can be evaluated using determinations of adenylate cyclase activity in membrane preparations of guinea pig mucosa after stimulation with histamine.

PROCEDURE

Preparation of membranes

Guinea pigs of either sex weighing 400–600 g are sacrificed. The fundic portion of the stomach is rapidly removed. Food particles are washed away with ice-cold 50 mM Tris buffer, pH 7.4, containing 4 mM EDTA and 0.25 M sucrose (homogenization buffer). The tissue is stretched, mucosal side up, on a glass Petri dish supported on ice and the mucosal layer is scraped off the muscle layer using a scalpel blade. The mucosal scrapings are transferred to 10 ml ice-cold homogenization buffer in a glass homogenization tube, and homogenized using an Ultra-Turrax® homogenizer followed by four strokes using a Potter Teflon homogenizer. The resulting suspension is centrifuged at 700 *g* for 10 min at 4 °C. The supernatant is discarded, and the pellet resuspended in 50 mM Tris buffer, pH 7.4, containing 4 mM EDTA followed by a further period of centrifugation. The resulting pellet is finally resuspended in Tris/EDTA buffer at a protein concentration of 2.4 mg/ml as determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Stimulation of adenylate cyclase

Adenylate cyclase activity is measured according to Hegstrand et al. (1976). Aliquots (120 µg) of fundic mucosal homogenate are incubated in 100 mM Tris buffer, pH 7.8, containing 0.6 mM EGTA (ethylene-glycol-bis-(β-aminoethyl ether)-N,N'-tetra-acetic acid), 1 mM IBMX (isobutyl-methyl xanthine), 2 mM $MgCl_2$, 0.1 mM GPT (guanosine triphosphate), histamine at various concentrations between 0.1 µM and 100 µM and the compound under investigation at varying concentrations between 0.1 µM and 1 mM for 10 min at 0 °C. The reaction is initiated by the addition of 1 mM ATP followed by a 10 min incubation at 30 °C. The reaction is terminated by placing the assay tubes in a boiling water bath for 3 min. After cooling, 40 mg of Alumina 90 (E. Merck, 70–230 mesh) are added to each tube prior to mixing and centrifugation at 700 *g* for 15 min at 4 °C.

Assay of cyclic AMP

The cyclic AMP content of each sample is determined according to Brown et al. (1971) using ^{3}H-cyclic AMP in a competitive protein binding assay. Fifty µl aliquots from the adenylate cyclase assay are incubated with 50 µl of ^{3}H-cyclic AMP (14 nC at a specific activity of 62 Ci/mmol) and 100 µl of previously prepared cyclic AMP binding protein at 4 °C. The bound and free ^{3}H-cyclic AMP are separated by the addition of 100 µl of charcoal reagent (2 g bovine serum albumin, 2.5 g Norit GSX charcoal in 100 ml of 50 mM Tris buffer, pH 7.4, containing 4 mM EDTA), followed by mixing and centrifugation at 1000 *g* for 15 min. Two hundred µl aliquots of the supernatant are decanted into scintillation vials containing 10 ml of NE 260 scintillator. Radioactivity is determined by liquid scintillation counting.

EVALUATION

Results are calculated from a standard curve using cyclic AMP as the standard. From these data IC_{50} values (µM) are derived.

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J.3.6.3 H^+/K^+-ATPase (proton pump) inhibition

J.3.6.3.1 General considerations

The parietal cell of the stomach is activated by three major stimuli: histamine, acetylcholine, and gastrin. In addition to the direct action of gastrin and acetylcholine on the parietal cell, these two agents may release histamine from a histamine storage in the gastric mucosa. In this manner, histamine would act as the final

mediator of acid secretion. One of the first events leading to acid secretion is a massive membrane transformation that occurs in the parietal cell. When the cell is stimulated, the tubulovesicles in the cytoplasma of the cell fuse and form an expanded secretory cannaliculus in the apical membrane where the enzyme H^+/K^+-ATPase is located.

In this way, the ultimate mediator of acid secretion in the stomach is the proton pump H^+/K^+-ATPase which transports hydrogen in exchange for potassium. The rat stomach H^+/K^+-ATPase has been cloned by Shull and Lingrel (1986). Development of specific inhibitors of this enzyme is an approach to suppress acid secretion and ulcer formation since ulcers only exist in acidic medium.

Alderuccio et al. (1993) described an experimental autoimmune gastritis in BALB/c mice as a $CD4^+$ T cell-mediated organ-specific autoimmune disease induced by neonatal thymectomy. Transgenic expression of the gastric H^+/K^+-ATPase β subunit specifically prevented the onset of this form of autoimmune gastritis.

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J.3.6.3.2 H^+/K^+-ATPase inhibition in membrane vesicles of stomach mucosa

PURPOSE AND RATIONALE

Several H^+/K^+-ATPase inhibitors contain a sulfhydryl group. At lower pH-values the compounds are protinated and rearrange to a sulphenic acid and a sulphenamide, that react with sulfhydryl groups in the enzyme. Therefore, the *in vitro* assays are performed both at neutral and at acidic pH levels.

PROCEDURE

Membrane vesicles containing H^+/K^+-ATPase are prepared from pig stomachs obtained from the local slaughter house (Ljungstrom et al. 1984). Pigs are fasted overnight before slaughter. The gastric mucosa of four stomachs is rinsed with cold saturated NaCl solution for 3–5 min. The superficial cells, cell debris plus the mucus are wiped off with the edge of a plastic ruler and with paper towels. The mucosa is scraped off. About 100 g scrapings are divided into portions of 10 g and homogenized in 0.25 M sucrose with seven strokes in a Potter-Elvehjem Teflon-glass homogenizer. The total volume is 600 ml which is centrifuged at 20 000 *g* for 40 min. The pellet is discarded. The supernatant is centrifuged at 75 000 *g* for 1 h. The resulting microsomal pellet is homogenized in 30 ml 0.25 M sucrose.

Aliquots of 15 ml are transferred to 100 ml centrifuge tubes and layered on top of step gradients, from the bottom comprising 25 ml 37% sucrose (w/v) and 45 ml 7.5% Ficoll (w/v) in 0.25 M sucrose. The tubes are centrifuged at 75 000 *g* for 1 h in a 6×100 ml ME angle rotor at 4 °C. The gradient is then fractionated by pumping Fluoroinert 70 through a narrow tubing in a fractionating cap down to the bottom of the tube. Fractions are collected from top through a center hole in the fractionating cap. The yield of vesicles in a typical preparation is about 50–75 mg protein. In order to maintain a stable vesicular structure for a long period of time, the vesicles are frozen at –70 °C under nitrogen. They can then be kept for several months without decrease of H^+/K^+-ATPase activity.

The ATPase activity is measured at 37 °C as the release of inorganic phosphate (P_i) from ATP. The test drug and the standard (omeprazole) are pre-incubated in concentrations of 0.01 to 100.0 µM in enzyme containing buffers in parallel at pH 6.0 and 7.4 for 30 min at 37 °C. Then, the medium of pH 6.0 is adjusted with HEPES/Tris buffer to pH 7.4. The enzyme reaction is started by addition of nigericin and Tris/ATP. The total reaction volume is 1 ml, containing 20 µg vesicular protein, 4 mM $MgCl_2$, 10 mM KCl, 20 µM nigericin, 2 mM Tris-ATP, 10 mM HEPES and additionally 2 mM Pipes for the pre-incubation medium at pH 6.0.

After 4 min at 37 °C, the reaction is stopped by the addition of 10 ml of 50% trichloroacetic acid. The denaturated protein is spun down and the P_i content is determined according to LeBel et al. (1978) based on the reduction of a phosphomolybdate complex by p-methyl-aminophenol sulfate in a copper acetate buffer or according to Carter and Karl (1982) based on the reaction of phosphomolybdate with the basic dye malachite green.

EVALUATION

IC_{50} values are calculated by probit analysis, whereby 0% corresponds to 4 mM Mg^{2+}-dependent and 100% to 4 mM Mg^{2+} plus 10 mM K^+-dependent ATP hydrolysis. IC_{50} values of the test compound at different pH values are compared with IC_{50} values of the standard. Statistical differences ($p < 0.05$) are calculated by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Proton transport in gastric vesicles can be measured by acridine orange fluorescence quenching (Lee and Forte 1978; Beil et al. 1990). Membrane protein (0.12 mg) is incubated at 37 °C in a volume of 2 ml containing: 10 mM Pipes/Tris buffer, pH 7.0 in the presence of 150 mM KCl, 2 mM $MgCl_2$, 2 mM ATP and 10 µM acridine orange. The pump reaction is started by the addition of valinomycine (ionophore for K^+). The decrease of fluorescence is studied at 530 mm as a measure for the intravesicular proton uptake.

CRITICAL ASSESSMENT OF THE METHOD

The pre-incubation period at the lowest possible pH of about 6 is used to initiate the acidic conversion of the test compound into its active principle. This reflects more the chemical instability of the test compound at neutral pH values than its effect during conditions of much higher acidity within the secretory cannaliculus of the parietal cell during acid secretion. Many chemically labile inhibitors are therefore very active in this test system. However, they do not cause an inhibition in more complex test systems and, therefore, are without any practical usefulness (Lindberg et al. 1990). Proton transport studies in gastric vesicles, using the acridine orange fluorescence quenching technique, where a pH gradient similar to *in vivo* conditions is formed (Lee and Forte 1978; Beil et al. 1990) are more suitable for studying the mechanism of action, acid-conversion, and structure-activity relationship of K^+/H^+-ATPase inhibitors (Herling and Weidmann 1994).

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J.3.6.3.3 Effect of H^+/K^+-ATPase inhibitors on serum gastrin levels

PURPOSE AND RATIONALE

It is known from the H^+/K^+-ATPase inhibitor omeprazole that the total acid blockade initiates a gastric antral feed back mechanism resulting in an excessive hypergastrinaemia (Arnold et al. 1986; Creutzfeldt et al. 1986; Larsson et al. 1986) which is believed to cause diffuse endocrine cell hyperplasia, characterized as carcinoids, in the gastric corpus after 2 years of treatment in the rat (Ekman et al. 1985).

PROCEDURE

Groups of 10–15 female Wistar rats weighing 90–110 g are treated daily for 10 weeks with omeprazole (10 or 30 mg/kg p.o.) or the test compound or serve as controls. The compounds are suspended in potato starch mucilage (20 mg/ml) and administered in a volume of 2 ml/kg. On days 1–3, the rats receive the H^+/K^+-ATPase inhibitors by intraperitoneal injection in order to cause gastric acid inhibition and therefore to reduce the gastric acid degradation of subsequent oral doses. After treatment for 2, 4, 7, and 10 weeks, blood samples are collected under ether anesthesia by retroorbital puncture. Gastrin is determined by radioimmunoassay using a commercially available kit, e.g. Gastrin RIAKit II; Dainabot Co., Ltd. At the end of the study

of 10 weeks, the animals are studied for their gastric acid output using the pylorus ligation (Shay technique).

EVALUATION

Serum gastrin levels are determined as pg/ml. Statistical differences ($p < 0.05$) are calculated using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Katz et al. (1987) described a five-day test to predict the long-term effects of gastric antisecretory agents on serum gastrin in rats.

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J.3.6.3.4 (^{14}C)-aminopyrine uptake and oxygen consumption in isolated rabbit gastric glands

PURPOSE AND RATIONALE

Isolated gastric glands from rabbits and other species can be used in studying the control of mechanism of gastric H^+ secretion (Berglindh and Öbrink 1967; Berglindh et al. 1967; Sack and Spenny 1982; Sewing et al. 1983; Herling et al. 1987, 1988, 1990). In isolated gastric glands and parietal cells, H^+ secretion cannot be directly measured by titration; therefore, accumulation of weak bases such as aminopyrine is used as an indirect probe of H^+ secretion. Moreover, glandular oxygen consumption can be measured with the Warburg technique.

PROCEDURE

Preparation of gastric glands

Rabbits are anesthetized with 40 mg/kg pentobarbital i.v. The abdomen is opened and the aorta is cannulated in a retrograde direction. Five ml of a heparin solution (250 IU/ml) are injected with force through the cannula. After one min the rabbit is bled through the cannula and a ligature is placed around the mesenteric vessels. The chest is quickly opened and the thoracic aorta clamped. Phosphate buffered saline solution (containing 149.6 mM NaCl, 3 mM K_2HPO_4, 0.64 mM NaH_2PO_4, pH 7.3) at 37 °C is pumped through the aorta, whereby the portal vein is opened to allow free outflow of the perfusate. By this procedure most of the solution is forced through the mesenteric vessels. The perfusion pressure, as measured proximal to the cannula, can be up to 600 mm Hg.

The stomach appears totally exsanguinated after perfusion with about 500 ml phosphate buffered saline solution and is then removed, cut open along the lesser curvature, and emptied. The cardial and antral regions are discarded. The corpus is rinsed several times with phosphate buffer solution and finally blotted with filter paper, whereby the remaining gastric content as well as some surface epithelial cells are removed. By blunt dissection the mucosa can easily be separated from the muscular and submuscular layers.

The mucosa is then minced into small pieces with a pair of scissors. The pieces are washed twice in warm oxygenated phosphate buffer solution and transferred to a 200 ml flask with 50 ml of a freshly prepared collagenase-enzyme solution containing 1 mg/ml collagenase (type I, Sigma), 1 mg/ml rabbit albumin (Sigma), 2 mg/ ml glucose in 130 mM NaCl, 10 mM $NaHCO_3$, 3 mM NaH_2PO_4, 3 mM Na_2HPO_4, 3 mM K_2HPO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, and 10 mg/L phenol-red at pH 7.4. The flask is gassed with 100% oxygen, sealed, and kept in a 37 °C water bath under gently stirring with a magnet for 90 min. The cloudy suspension containing the separated glands and some cells is filtered through a nylon mesh into 15 ml test tubes with conical bottoms. The glands rapidly sediment to the bottom while the free cells remain in the solution. In this way centrifugation can be avoided and the glands can easily be washed free from isolated cells and collagenase by three washings with phosphate buffer at room temperature.

^{14}C-aminopyrine accumulation in gastric glands

The ability of gastric glands to form acid is measured based on aminopyrine accumulation. The glands are diluted to a final concentration of 2–4 mg dry weight/ml in a medium containing 100.0 mM NaCl, 5.0 mM KCl, 0.5 mM NaH_2PO_4, 1 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20.0 mM $NaHCO_3$, 20.0 mM HEPES, 2 g/ml glucose, 1 mg/ml rabbit albumin, adjusted with 1 M Tris to pH 7.4. Samples of 1 ml gland suspension are equilibrated in 1 ml medium containing 0.1 µCi/ml [^{14}C]aminopyrine at 37 °C in a shaking water bath to-

gether with the agent to be tested. After 20 min either histamine or dibutyryl-cyclic-AMP is added, followed by a 30 min incubation period for histamine and a 45 min incubation period for dibutyryl-cyclo-AMP.

The glands are then separated from the medium by brief centrifugation. The supernatant is withdrawn, the pellets are dried at 80 °C for 50 min, weighed and dissolved in 200 µl 1 M NaOH. Aliquots of the supernatant and the digested gland pellet are examined in a liquid scintillation counter. The ratio of intraglandular to extraglandular radioactivity is calculated. All determinations are made in triplicate.

Respiratory studies

Glandular oxygen consumption is measured at 37 °C using a Warburg respirometer and air as the gas phase. The 15 ml flasks with a central well 20% containing KOH solution on a filter paper as CO_2 adsorber are filled with 1 ml medium containing 100.0 mM NaCl, 5.0 mM KCl, 0.5 mM NaH_2PO_4, 1 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20.0 mM $NaHCO_3$, 20.0 mM HEPES, 2 g/ml glucose, 1 mg/ml rabbit albumin, adjusted with 1 M Tris to pH 7.4 and 1 ml gland suspension. After 20 min equilibration, the test compound and dbcAMP are added and the oxygen consumption is measured at 15 min intervals for the following 45 min. The recorded oxygen consumption is corrected according to the following formula:

$$O_2 \text{ consumption} = O_2 \text{ recorded} \times K$$

where

- $K = 273 \times (Pb / t + 273) \times 760$
- t = ambient temperature in °C around the manometers
- Pb = Atmospheric pressure (mm Hg)

The respiratory activity is expressed in µl O_2 consumed per mg dry weight and time.

EVALUATION

IC_{50} values are calculated by probit analysis. Statistical differences ($P < 0.05$) are assessed by Student's t-test, n = the number of different gland preparations.

CRITICAL ASSESSMENT OF THE METHOD

Studies on (^{14}C)-aminopyrine uptake and oxygen consumption in isolated rabbit gastric glands have become a valuable approach for studying the effect of various H_2-receptor antagonists and proton pump inhibitors. When studying gastric acid production with the ^{14}C-AP accumulation technique, the addition of basic drugs can be problematic (Fryklund and Wallmark 1986). The basic nature of a test compound can compete with accumulation of ^{14}C-AP. Nevertheless, it is generally accepted that oxygen consumption of gastric glands correlates well with acid formation (Berglindh et al. 1976).

MODIFICATIONS OF THE METHOD

Soll (1978) studied the actions of secretagogues on oxygen uptake by isolated mammalian parietal cells. Parietal cells were prepared from the stomach of dogs by collagenase digestion and counterflow centrifugation. Oxygen consumption was determined by polarography. Isobutyl methyl xanthine (IMX), carbamylcholine, histamine, and gastrin each independently stimulated oxygen uptake. The specificity of these responses was tested by use of an H_2-histamine receptor antagonist or atropine as anticholinergic agent.

Stoll (1980) investigated the [^{14}C]aminopyrine accumulation in isolated *canine* parietal cells when treated with histamine, gastrin and carbachol and the displacement by cimetidine or atropine.

Sewing et al. (1983, 1986) studied the effect of several benzimidazole derivatives on isolated and enriched guinea pig parietal cells using the ^{14}C-aminopyrine accumulation.

Schepp et al. (1994) determined the effects of exendin-4, a peptide from *Heloderma suspectum venom*, and exendin-(9-39)NH_2 on [^{14}C]aminopyrine accumulation in isolated rat parietal cells and compared these with those of glucagon-like peptide-1-(7-36)NH_2.

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J.3.6.3.5 Gastric mucosal blood flow

PURPOSE AND RATIONALE

Hydrogen gas clearance has been used to measure blood flow in the basal portion of gastric mucosa in anesthetized rats (Leung et al. 1984, 1986; Pique et al. 1988).

PROCEDURE

Male Sprague Dawley rats are anesthetized with 1.5 g/kg of urethane subcutaneously. A tracheotomy is performed and a PE-250 tubing is inserted into the trachea to facilitate spontaneous breathing and for administration of 3% hydrogen in air. The right carotid artery is cannulated for blood pressure monitoring. A midline laparatomy is then performed and the stomach exteriorized. Through an incision in the forestomach the gastric contents are gently washed out with physiological saline. A double-lumen cannula (outer: Tygon with a diameter of 7 mm; inner: polyethylene with a diameter of 2 mm) are inserted into the stomach and secured by a ligature at the forestomach. The pylorus is ligated and a 0.9% sodium chloride solution is infused through the inner cannula at a rate of 0.8 ml/min and drained from the outer tubing. The gastric effluent is collected at 15-min intervals. Acid output (in micro-equivalents per min) is determined in the perfusate with 0.2 N NaOH with an automatic titrator.

An incision in the serosa and the muscularis externa is made in the gastric wall of the corpus, exposing 3–4 mm of the submucosa. Through this hole a platinum electrode is placed in contact with the exposed basal portion of the mucosa. The electrode is made with a ring of platinum wire, 125 µm in diameter, wound around a glass capillary tube and held in place inside a Teflon tube by epoxy. A Ag-AgCl reference electrode is placed inside the peritoneal cavity. The laparatomy incision is covered by Parafilm to minimize evaporation.

One femoral vein is cannulated for the infusion of saline or drugs. The rat is kept warm with a heat lamp to maintain rectal temperature at 37 °C.

Hydrogen gas clearance technique

Current is generated at the surface of a platinum electrode by oxidation of molecular hydrogen to hydrogen ions and electrons. This current, which is measured using a polarographic and amplifying unit, is proportional to the hydrogen tension gradient at the surface of the platinum electrode. When the experimental animal breathes 3% hydrogen in air, the current tracing, graphed on a recorder, gradually rises and reaches a plateau as the tissue adjacent to the electrode is saturated with hydrogen. After the external hydrogen source is removed, the current tracing gradually falls because of removal of tissue hydrogen by blood flow. The rate of dissipation of hydrogen estimates the tissue blood flow (Aukland et al. 1964).

The exponential decrease of the hydrogen gas clearing curves is evaluated by a computer program (Livingstone et al. 1986). Current from the platinum electrode is passed through an ADALAB analog-to-digital converter and discrete digitized values are sampled every 5 s. Blood flow is determined by an Newtonian-Gaussian nonlinear iterative regression program by means of a biexponential formula:

$$f(x) = A + B\, e^{-k1t(t-T)} + B\, e^{-k2t(t-T)},$$

where $f(x)$ is the electrode current, A is the baseline current, B is the initial current, e is the base of natural logarithms, k_1 is the rate constant for the fast component, k_2 is the rate constant for the slow component, t is the time at which the current is observed, and T is the time when hydrogen gas was removed.

Mucosal blood flow is expressed in milliliters per minute per 100 g of tissue.

Inhibition of acid secretion

Intravenous saline is infused during the first 45 min of the study. After this period, an infusion of 80 µg/kg·h of pentagastrin is administered for 135 min. During the last 75 min an intravenous infusion of the inhibitor or vehicle is administered simultaneously with pentagastrin. Corpus mucosal blood flow measurements are obtained during the resting period, 45 min after start for the pentagastrin infusion, and during the last 15 min of combined infusion of pentagastrin and inhibitor. Acid output is measured at 15 min intervals throughout the experiments.

EVALUATION

All data are expressed as mean ±standard error. The data are analyzed using a paired *t*-test for comparison

of basal versus stimulated condition within the same animal, analysis of variance with contrasts, and linear and polynomial regression analysis for comparison between animals in different groups. A probability level of $P < 0.05$ is considered significant.

MODIFICATION OF THE METHOD

The hydrogen gas clearance technique has been used by many authors in experimental gastroenterology, e.g.: Hirose et al. (1991), Holzer and Guth (1991), Lippe and Holzer (1992), Pique et al. (1992), Tsukamoto et al. (1992), Lazaratos et al. (1993), Petho et al. (1994), Tanaka and Guth (1994), Goldin et al. (1996), Hisanaga et al. (1996), Doi et al. (1998), Heinemann et al. (1999).

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J.3.7 Anti-ulcer activity

J.3.7.1 Pylorus ligation in rats (SHAY rat)

PURPOSE AND RATIONALE

A simple and reliable method for production of gastric ulceration in the rat based on ligature of the pylorus has been published by Shay et al. (1945). The ulceration is caused by accumulation of acidic gastric juice in the stomach.

PROCEDURE

Female Wistar rats weighing 150–170 g are starved for 48 h having access to drinking water ad libitum. During this time they are housed single in cages with raised bottoms of wide wire mesh in order to avoid cannibalism and coprophagy. Ten animals are used per dose and as controls. Under ether anesthesia a midline abdominal incision is made. The pylorus is ligated, care being exercised that neither damage to the blood supply nor traction on the pylorus occurs. Grasping the stomach with instruments is to be meticulously avoided, else ulceration will invariably develop at such points. The abdominal wall is closed by sutures. The test compounds are given either orally by gavage or injected subcutaneously.

The animals are placed for 19 h in plastic cylinders with an inner diameter of 45 mm being closed on both ends by wire mesh. Afterwards, the animals are sacrificed in CO_2 anesthesia. The abdomen is opened and a ligature is placed around the esophagus close to the diaphragm. The stomach is removed, and the contents are drained in a centrifuge tube. Along the greater curvature the stomach is opened and pinned on a cork plate. The mucosa is examined with a stereomicro-

scope. In the rat, the upper two fifths of the stomach form the rumen with squamous epithelium and possess little protective mechanisms against the corrosive action of gastric juice. Below a limiting ridge, in the glandular portion of the stomach, the protective mechanisms are better in the mucosa of the medium two fifths of the stomach than in the lowest part, forming the antrum. Therefore, lesions occur mainly in the rumen and in the antrum. The number of ulcers is noted and the severity recorded with the following scores:

- 0 = no ulcer
- 1 = superficial ulcers
- 2 = deep ulcers
- 3 = perforation.

The volume of the gastric content is measured. After centrifugation, acidity is determined by titration with 0.1 n NaOH.

EVALUATION

An ulcer index U_I is calculated:

$$U_I = U_N + U_S + U_P \times 10^{-1}$$

- U_N= average of number of ulcers per animal
- U_S = average of severity score
- U_P = percentage of animals with ulcers

Ulcer index and acidity of the gastric content of treated animals are compared with controls. Using various doses, dose-response curves can be established for ulcer formation and gastric acid secretion. ID_{50} values can be calculated by probit analysis, whereby 0% corresponds to no and 100% to maximal stimulated gastric acid output.

CRITICAL ASSESSMENT OF THE METHOD

The "Shay-rat" has been proven to be a valuable tool to evaluate anti-ulcer drugs with various mechanisms of action.

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J.3.7.2 Stress ulcer through immobilization stress

PURPOSE AND RATIONALE

Psychogenic factors, such as stress, play a major role in the pathogenesis of gastric ulcers in man. The first report of the use of restraint as stress factor was published by Selye (1936). Hanson and Brodie (1960) and Bonfils et al. (1966) described methods to study the effect of anti-ulcer drugs on immobilization stress in rats.

PROCEDURE

Groups of 10 female Wistar rats per dose of test drug and for controls weighing 150–170 g are used. Food and water are withdrawn 24 h before the experiment. After oral or subcutaneous administration of the test compound or the placebo solution the animals are slightly anesthetized with ether. Both lower and upper extremities are fixed together and the animals are wrapped in wire gaze. They are horizontally suspended in the dark at 20 °C for 24 h and finally sacrificed in CO_2 anesthesia. The stomach is removed, fixed on a cork plate and the number and severity of ulcers is registered with a stereo-microscope using the following scores:

- 0 = no ulcer
- 1 = superficial ulcers
- 2 = deep ulcers
- 3 = perforation

EVALUATION

An ulcer index U_I is calculated:

$$U_I = U_N + U_S + U_P \times 10^{-1}$$

- U_N = average of number of ulcers per animal
- U_S = average of severity score
- U_P = percentage of animals with ulcers

CRITICAL ASSESSMENT OF THE METHOD

The experimental model resembles the psychogenic factors in the pathogenesis of gastric ulcers in patients. Therefore, it is not surprising that not only antacids, anticholinergics, H_2-antagonists, proton-pump-inhibi-

tors, but also psychotropic drugs, like neuroleptics, have been found to be effective in this test. The test is being used in final drug evaluation only.

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J.3.7.3 Stress ulcers by cold water immersion

PURPOSE AND RATIONALE

Cooling of rats in water during the restraint period accelerates the occurrence of gastric ulcers and shortens the time of necessary immobilization (Takagi et al. 1964; West 1982).

PROCEDURE

Groups of 8–10 Wistar rats weighing 150–200 g are used. After oral administration of the test compound, the rats are placed vertically in individual restraint cages in water at 22 °C for one hour. Then, they are removed, dried and injected intravenously via the tail vein with 30 mg/kg Evans blue. Ten min later, they are sacrificed in CO_2 anesthesia and their stomachs removed. Formol-saline (2% v/v) is then injected into the totally ligated stomachs for storage overnight. The next day, the stomachs are opened along the greatest curvature, washed in warm water, and examined under a 3-fold magnifier. The lengths of the longest diameters of the lesions are measured and summated to give a total lesion score (in mm) for each animal, the mean count for each group being calculated.

EVALUATION

The mean score in control rats is about 25 (range 20–28). Inhibition of the lesion production is expressed as percentage value.

CRITICAL ASSESSMENT OF THE METHOD

Like other stress models, the test resembling the psychogenic factor for ulcer disease in human beings, is used for final drug evaluation only.

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J.3.7.4 Indomethacin induced ulcers in rats

PURPOSE AND RATIONALE

Nonsteroidal anti-inflammatory agents, like indomethacin and acetyl-salicylic acid, induce gastric lesions in man and in experimental animals by inhibition of gastric cyclo-oxygenase resulting in less formation of prostacyclin, the predominant prostanoid produced in the gastric mucosa.

PROCEDURE

Groups of 8–10 Wistar rats weighing 150–200 g are used. The test drugs are administered orally in 0.1% Tween 80 solution 10 min prior to oral indomethacin in a dose of 20 mg/kg (4 mg/ml dissolved in 0.1% Tween 80 solution). Six hours later, the rats are sacrificed in CO_2 anesthesia and their stomachs removed. Formol-saline (2% v/v) is then injected into the totally ligated stomachs for storage overnight. The next day, the stomachs are opened along the greater curvature, then washed in warm water, and examined under a 3-fold magnifier. The lengths of the longest diameters of the lesions are measured and summated to give a total lesion score (in mm) for each animal, the mean count for each group being calculated.

EVALUATION

The mean score in control rats is about 25 (range 20–28). Inhibition of the lesion production is expressed as percentage value.

MODIFICATION OF THE METHOD

Dose- and time dependency of the ulcerogenic action of indomethacin were studied by Djahanguiri (1969).

Instead of indomethacin, gastric lesions can be induced by intravenous or oral doses of aspirin which can be prevented by exogenous PGE_2 or PGI_2 (Konturek et al. 1981). Furthermore, reserpine at a dose of 8 mg/kg i.p., or cysteamine hydrochloride at a dose of 400 mg/kg s.c. was given in order to induce ulcers in rats (Tarutani et al. 1985).

Kitajima et al. (1993) studied the role of endothelin and platelet-activating factor in indomethacin-induced gastric mucosal injury in rats. Four hours after subcutaneous injection of 25 mg/kg indomethacin, the rats

were sacrificed after ether anesthesia, and the stomach was removed. The stomach was filled with 1.5 ml of 2% buffered formalin for 10 min and then opened along the greater curvature. The total length of the lesions was measured.

Wallace et al. (1989) studied the ulcerogenic activity of endothelin in indomethacin pretreated rats using an *ex vivo* gastric chamber.

Scarpignato et al. (1995) evaluated NSAID-induced gastric mucosal damage by continuous measurement and recording gastric potential difference in the rat.

CRITICAL ASSESSMENT OF THE METHOD

According to West (1982) the cold stress induced ulcer formation, but not the indomethacin- or aspirin-induced ulcers are inhibited by H_2-receptor antagonists, whereas other authors reported protective effects of H_2-receptor antagonists under these conditions (Tarutani et al. 1985).

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J.3.7.5 Ethanol induced mucosal damage in rats (cytoprotective activity)

PURPOSE AND RATIONALE

Intragastric application of absolute ethanol is a reproducible method to produce gastric lesions in experimental animals (Robert et al. 1979; Szabo et al. 1981). These lesions can be at least partially inhibited by various drugs, such as some prostaglandins. The protective effect against various irritants has been called cytoprotective activity (Robert 1979; Robert et al. 1979). The method has been modified by several authors. Witt et al. (1985) described a method to objectively quantify the extent of ethanol-induced gastric lesions utilizing a transmission densitometer to measure the optical density of the photographic negative of the stomach mucosa.

PROCEDURE

Male Wistar rats weighing 250–300 g are deprived of food 18 h prior to the experiment but are allowed free access to water. During this time they are kept in restraining cages to prevent coprophagy. The rats are administered either the appropriate vehicle or the cytoprotective drug, e.g. a prostanoid, intragastrally 30 min prior to administration of 1 ml absolute ethanol. Untreated animals are included as controls. One hour after administration of ethanol, the animals are euthanized with CO_2, the stomachs are excised, cut along the greater curvature, and gently rinsed under tap water. The stomachs are stretched on a piece of foam core mat, mucosal site up.

The subjective scores of the treated tissues are recorded; the graded response is reflecting the least (0) to most (3) damage. A circular full thickness area, about 13 mm in diameter, is cut with a cork borer from each lobe of the fundus just below the ridge dividing the glandular from the non-glandular portion of the stomach. A Plexiglas template ($19 \times 14 \times 0.3$ cm), burnished on one side with emery cloth, and with four rows with six holes 13 mm in diameter is placed on a sheet of clear glass, burnished side up, and bound to the glass with photographic tape along the periphery. The excised pairs of tissue from each stomach are placed into the holes of the template.

Pairs of tissue from each stomach are examined to minimize sampling errors. The template is positioned on a rectangular central open area of an Aristo Model T-16 cold cathode transilluminator (38×38 cm) containing a W-45 blue-white lamp. A camera is mounted on a copy stand directly above the template. Photographs are taken, the film processed in a standard manner and a contact sheet is made from the negatives. A light transmission densitometer (e.g. MacBeth model TD-501) is used to evaluate the negatives. The optical density of the test tissues is determined by placing each area of the negative in sequence over the aperture through which the light is transmitted. The optical density is displayed on a digital read out and recorded. Hemorrhagic or damaged areas appear bright on the negative, whereas undamaged tissue appears dark. Hence, lower optical density values are indicative of

damage while higher optical densities are associated with little, or, as in the case of control, no damage.

EVALUATION

The significance of differences in optical density between control and ethanol-treated tissue is evaluated by nonpaired single-tail Student's *t*-test.

MODIFICATIONS OF THE METHOD

Cytoprotection by prostaglandins was studied in rats by prevention of gastric necrosis produced by various agents such as alcohol, HCl, NaOH, hypertonic NaCl, and thermal injury (Robert et al. 1979) and against gastric injury produced by nonsteroidal anti-inflammatory compounds (Robert 1979; Franzone et al. 1988). The animals are fasted 48 h prior to the experiment and placed 18 h before the administration of drugs into plastic tubes to prevent coprophagy. Fifteen min after application of the test drug, the animals are given 1 ml of the irritant orally. After an additional hour, the animals are sacrificed, the stomachs removed and immediately opened along the greater curvature. Lesions are counted and scored (0 = no lesion; 1 = mild lesions; 2 = severe lesions; 4 = necrosis).

Starrett et al. (1989) employed 3.0 ml/kg ethyl alcohol (100%) or 3.0 ml/kg 0.75 N HCl as necrotizing agent.

Borella et al. (1989) studied the cytoprotective and anti-ulcer activities of the anorganic antacid Magaldrate in the rat using absolute ethanol as irritant.

Masuda et al. (1993) investigated the role of endogenous endothelin in the pathogenesis of ethanol-induced gastric mucosal injury in rats.

CRITICAL ASSESSMENT OF THE METHOD

Several prostaglandins provide cytoprotection, particularly in rats, in a dose-range which has no antisecretory activity. However, clinical experience with prostaglandins showed that ulcer healing is only achieved at antisecretory doses (Lindberg et al. 1990). Therefore, it seems very likely that the cytoprotective property of a compound in rats has very limited relevance to prediction of its ulcer healing potential in humans if cytoprotection is really separated from its antisecretory potential (Herling and Weidmann 1994).

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J.3.7.6 Subacute gastric ulcer in rats

PURPOSE AND RATIONALE

Ezer (1988) described a method for producing standard subacute gastric ulcers in rats and for the quantitative evaluation of the healing process.

PROCEDURE

Female Wistar rats weighing 120–150 g are fasted for 24 h having access to water at libitum in cages with wire sieves at the bottom. The rats are anesthetized with ether and a polyethylene catheter including a fine steel wire with a needle tip (1.2 mm diameter) at the lower end is orally inserted into the stomach. After the cannula reaches the gastric wall, the upper end of the steel wire is pressed in a definitive manner, so as to puncture the gastric wall. Each rat is kept in the same position during the intervention in order to localize the puncture at nearly the same region of the glandular part of the stomach. The test substances are administered orally, 30 min or 24 h after puncture. Free access to food and water is provided from 2 h up to the end of the experiment. Each group consists of 8–15 rats.

The animals are sacrificed by overdose of ether at definitive time intervals after puncture. The stomach is dissected and opened along the lesser curvature, extensively rinsed in tap water and fixed to the end of a

polyethylene tube of 10 mm diameter (plastic tip of an automatic pipette) in a position with the punched ulcer in the center. The end of the tube with the gastric wall is suspended in a beaker containing physiological saline, and the pressure in the tube is gradually increased with a valved rubber ball connected to the other end of the tube. The third part of the system is a tonometer calibrated up to 1 bar. The value of tension at which bubbles appear at the ulcerous gastric wall is noted. This value is termed as tensile strength and can be expressed in mm Hg.

EVALUATION

The extent of the healing of gastric ulcers can be characterized by the healing rate (*HR*) according the following equation:

$$HR = (A - B) / C \quad (\text{mm Hg/h})$$

with

- A = tensile strength (mm Hg) at C time-point after puncture
- B = tensile strength 30 min after puncture (the average value is 143 mm Hg)
- C = time course (h) of the experiment.

Anti-ulcer drugs, such as H_2 antagonists, significantly increase the healing rate, which is decreased by non-steroidal anti-inflammatory drugs.

CRITICAL ASSESSMENT OF THE METHOD

Similarly to the method of Takagi et al. (1969) who injected 50 µl of acetic acid into the stomach wall (Szelenyi et al. 1982), the method of Ezer (1988) allows to judge the time course of healing of the ulcers.

MODIFICATIONS OF THE METHOD

Okabe and Pfeiffer (1972) induced chronic gastric ulcer in rats by temporary instillation of acetic acid. In pentobarbital anesthesia, a cylindrical glass tube of 6 mm in diameter was tightly placed upon the anterior serosal surface of the glandular portion of the stomach one cm away from the pyloric end. A dose of 0.06 ml/animal of 50% acetic acid was instilled into the tube and allowed to remain one min on the gastric wall. After removal of the acid solution, the abdomen was closed in two layers and the animals brought back to their cages and fed normally. Test drugs were given orally on day 1 twice daily, 4 h after application of acetic acid and continued up to 10 days after induction of ulcer. The animals were sacrificed after 18 h of the last dose to assess ulcer size and healing. Ulcer index was calculated upon the product of length and width of ulcers.

Karmeli et al. (1996) induced gastric mucosal erosions in rats by addition of 0.1% iodoacetamide to the drinking water. The animals were sacrificed after various time intervals, the stomach was resected, washed, lesion area assessed, and mucosal inflammatory mediators determined. Myeloperoxidase was increased and nitric oxide synthase activity decreased. The damage induced by iodoacetamide was significantly ameliorated by treatment with a free radical scavenger, (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl, TEMPOL).

Konturek et al. (1999) described a mouse model of *Heliobacter pylori* infection. Gastric function and healing of chronic acetic acid-induced ulcers in BALB/c mice were studied after inoculation with CagA and VacA positive (type I) or CagA and VacA negative (type II) *Heliobacter pylori* strains. This infection caused immediate suppression of gastric secretion and delayed the healing of ulcers.

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J.3.7.7 Gastric ischemia-reperfusion injury in rats

PURPOSE AND RATIONALE

Hassan et al. (1997) described the effect of an endothelin converting enzyme inhibitor on local gastric ischemia-reperfusion injury in rats. Endothelin-1 has potent ulcerogenic effects in the stomach (Wallace et al. 1988). Endogenous endothelin-1 has been implicated for ethanol-, indomethacin- and hemorrhagic shock-induced gastric ischemia-reperfusion injuries (Masuda et al. 1993; Kitajima et al. 1993; Michida et al. 1994; Kitajima et al. 1995).

PROCEDURE

Male Wistar rats weighing 200–250 g are fasted for 24 h with free access to water. The rats are anesthetized with 1.5 g/kg urethane i.p. The stomach is exposed by a medial laparatomy and instilled with 0.15 M HCl (1 ml/100 g) via the forestomach. The left gastric artery is clamped by a small vascular clamp for 5 min to induce ischemia and 30 min of reperfusion is done by releasing the clamp. Pretreatment with test drug or standard is given to groups of 5 rats immediately before the induction of ischemia. At the end of the experiment, the rats are sacrificed by cervical dislocation. The stomach is fixed with 10% buffered formalin and photographed for macroscopic evaluation of injuries. For the assessment of microscopic injuries, a sample of corpus 0.5 cm below the limiting ridge containing the entire width of the anterior wall is taken from each stomach and processed for subsequent histological evaluation.

A planimeter attached to a computer is used to trace the macroscopic mucosal injury from color photographs. The results are expressed as a percentage of the total glandular mucosal area.

Each histological section is stained with hematoxylin/eosin and examined under light microscope. An one cm length of each histological section is assessed for epithelial damage (score = 1), glandular disruption, vasocongestion or edema in the upper mucosa (score = 2), hemorrhagic damage in the mid to lower mucosa (score = 3) and deep necrosis and ulceration (score = 4). Each section is evaluated on a cumulative basis to give the histological index, the maximum score thus being 10.

EVALUATION

Data are expressed as mean ±SEM. Comparisons between different groups are made by one way analysis of variance followed by Fisher's least significant difference test. P-values of <0.05 are considered as statistically significant.

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J.4 Intestinal functions

J.4.1 Intestinal secretion

J.4.1.1 Laxative activity in rats

PURPOSE AND RATIONALE

Laxatives of the sennoside type act mainly by acceleration of large intestine transit and inhibition of fluid absorption in the colon (Leng-Peschlow 1986).

PROCEDURE

For **measurement of large intestinal transit time**, female Wistar rats weighing approximately 200 g are anesthetized with ether. A PVC catheter is implanted into the caecum with the distal end fixed on the animal's neck. The animals are allowed to recover and are placed individually in a wire meshed cage to enable the feces to fall through onto blotting paper. Carmine red (10 mg in 0.4 ml distilled water per animal) is injected through the catheter immediately after administration of the test substance. The time until appearance of the first colored feces is registered.

For **measurement of fluid absorption in the colon**, female Wistar rats weighing approximately 200 g are anesthetized with 50 mg/kg pentobarbitone sodium. The colon is ligated and cannulated distal to the caecocolic junction (PE-tube, i.d. 1 mm) and, after a thorough rinse with 50 ml physiological saline to remove all contents, a second cannula (silicone, i.d. 3 mm) is inserted proximal to the rectum for fluid outflow. Four and 6 h after oral administration of the test compounds an open perfusion with an electrolyte solution (NaCl 6.72 g/l, KCl 0.37 g/l, $NaHCO_3$ 2.1 g/l, polyethylene glycol (PEG, mol wt 4 000) 2.0 g/l, [^{14}C]PEG 5 µCi/l; pH 6.5, osmolality 275 milliosmol/kg) is started at a rate of 12 ml/h for two consecutive 2 h periods. [^{14}C]PEG activity is measured by liquid scintillation counting, Na^+ and K^+ by flame photometry, Cl^- by coulometric titration, osmolality by freezing point depression and mucus as protein-bound total hexoses by the orcinol-sulphuric acid method. Net H_2O, Na^+, K^+ and Cl^- transport are calculated and expressed as ml or µmol/h and per 10 cm colon length.

EVALUATION

All values are expressed as mean ± standard deviation. Statistical significance is assessed with Student's *t*-test.

MODIFICATIONS OF THE METHOD

Ogunti and Elujoba (1993) tested the laxative activity of *Cassia alata* in rats. Male Charles River rats were kept in individual cages during one week. Any rat producing wet feces was rejected. After administration of the test compounds to groups of 5 rats per dose, the feces were examined for wetness hourly for 12 h. The results were expressed as the mean percent of total feces that were wet per kg rat.

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J.4.1.2 Enteropooling test

PURPOSE AND RATIONALE

The enteropooling assay in rats has been developed by Robert et al. (1976) to test the diarrheogenic property of prostaglandins for prediction of this clinically relevant side effect of several synthetic prostaglandins.

PROCEDURE

Female Sprague Dawley rats weighing 190–215 g are used. The animals are fasted overnight having free access to water. The test compounds are administered orally, and the animals, 12 per group, are sacrificed one hour later. The fluid accumulation occurs in the small intestine which is cut at the pylorus and the ileocecal junction, and its contents, consisting of a thick fluid (in controls) and a very watery fluid (in prostaglandin-treated animals) are collected into a graduated test tube by milking the whole length of the small intestine with the fingers. The volume of fluid is recorded.

EVALUATION

Using various doses, dose-response curves can be established and potency ratios calculated. 16,16-dimethyl PGE_2 was found to be the most active compound.

CRITICAL ASSESSMENT OF THE METHOD

Some other diarrheogenic agents, like $MgSO_4$, castor oil, bile, taurocholate and taurochenodesoxy-cholate cause enteropooling, whereas mineral oil and tragacanth are ineffective. The anticholinergic agent methylscopolamine partially counteracted the enteropooling. The assay, therefore, can be used to test the laxative or the antidiarrheal activity of compounds (Shook et al. 1989).

MODIFICATIONS OF THE METHOD

Beubler and Badhri (1990) used the PGE_2-induced net fluid secretion in the jejunum and colon in the rat to evaluate the antisecretory effects of antidiarrheal drugs. Polyethylene catheters were placed into the jejunum and colon and Tyrode solution was instilled into the loops. Net fluid transfer rates were determined gravimetrically 30 min after instillation of Tyrode solution.

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J.4.1.3 Inhibition of chloride secretion in rabbit colon[2]

PURPOSE AND RATIONALE

Epithelia have the ability to reabsorb or to secrete fluids and electrolytes. In 1958, Koefoed-Johnsen and Ussing first published a model of ion transport across frog skin epithelium. Ussing also introduced the measurement of the short-circuit current as a means of defining active ion transport (Koefoed-Johnsen and Ussing 1958). Mammalian colon is an example of epithelium which has the capacity to absorb and to secrete electrolytes.

Previous studies have shown that mammalian colon actively secretes chloride when exposed to prostaglandins and vasoactive intestinal peptides, which increase the cellular concentration of cAMP resulting in an electrogenic chloride secretion (Frizzell et al. 1976). The elevation of cAMP causes the opening of chloride channels, whereas the colonic epithelium absorbs sodium and chloride ions by an electroneutral mechanism under control conditions. In general it is assumed that absorption takes place mainly in the surface cells, whereas the crypts are the predominant site of secretion (Greger et al. 1985; Binder and Sandle 1987).

[2] Contributed in the first edition by M. Hropot.

PROCEDURE

Rabbits of either sex (2.0 to 4.0 kg body weight) are killed by cervical dislocation. The distal colon is removed, immediately opened into a flat sheet and washed in the standard electrolyte solution. The epithelium with an area of 1.0 cm^2 is stripped from its underlying musculature and mounted in an Ussing chamber. The tissue is mounted vertically and bathed on both sides by electrolyte solutions, which are circulated and oxygenated by a water-jacketed bubble-lift apparatus maintained at 37 °C. The carbogen gas used for bubbling contains O_2 and CO_2 in a mixture of 95% and 5%, respectively. The solutions used on the two sides have the following composition (in mmol/l): NaCl 120, $NaHCO_3$ 21, Na_2HPO_4 0.4, K_2HPO_4 1.6, $MgCl_2$ 1.2, glucose 5.

Tissues are continuously short-circuited by a four-electrode automatic voltage-clamp apparatus (AC-Microclamp, Aachen, Germany) which measures short-circuit current (I_{sc}) and automatically subtracts chamber fluid resistance. Transepithelial electrical potential difference is measured between Ag-AgCl electrodes, which make contact with the bathing solutions via agar bridges (5% agar in glucose-free standard electrolyte solution). I_{sc} is measured by passing sufficient current through Ag-AgCl electrodes to reduce the spontaneous transepithelial electrical potential difference to zero. Transepithelial conductance (G_t) is determined by passing 100-ms bipolar current pulses through the tissue.

The standard protocol consists of an initial equilibration period of 20 min. The I_{sc} measured in the presence of indomethacin (1 µmol/l, serosal and mucosal solution) corresponds mostly to the rheogenic reabsorption of sodium. In the next step, amiloride is added at 0.1 mmol/l to block sodium reabsorption. Then PGE_2 (1 µmol/l) is added to the serosal solution in the presence of amiloride in order to stimulate chloride secretion. After a further equilibration period of 20 min putative blockers of chloride secretion are added to the mucosal or serosal solution.

EVALUATION

Each compound is examined at least 3 times and at three different concentrations (usually 1, 10 and 100 µmol/l). From the mean values a concentration-response curve is constructed, and from this curve the IC_{50} value is read as the concentration producing 50% inhibition of the stimulated I_{sc}.

Data are presented as means ±SEM. Paired *t*-test with a significance level of $p < 0.05$ may be used.

CRITICAL ASSESSMENT OF THE METHOD

The method can be used to study the antidiarrhoic activity of a test compound, but also generally its influence on active electrolyte transport across the cell membrane.

MODIFICATIONS OF THE METHOD

Warhurst et al. (1996) studied the effects of somatostatin analogues on electrogenic ion secretion in isolated rat colonic mucosa mounted in Ussing chambers.

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J.4.2 Antidiarrhea effect

J.4.2.1 Castor oil induced diarrhea

PURPOSE AND RATIONALE

The induction of diarrhea with castor oil results from the action of ricinoleic acid formed by hydrolysis of the oil (Iwao and Terada 1962; Watson and Gordon 1962). Ricinoleic acid produces changes in the transport of water and electrolytes resulting in a hypersecretory response (Ammon et al. 1974). In addition to hypersecretion, ricinoleic acid sensitizes the intramural neurons of the gut.

PROCEDURE

Female Wistar rats weighing 210–230 g are used after overnight food deprivation. For the experiment, the rats are housed in individual cages with no access to drinking water. The potential antidiarrhoic agents are administered orally by gavage in various doses. Controls receive the solvent only. Each dose is given to 10 animals. One hour after dosage, 1 ml of castor oil is administered orally. Stools are collected on non-wetting paper sheets of uniform weight up to 24 h after administration of the castor oil. Every 15 min during the first 8 h, urine is drained off by gravity, and the net stool weight, termed early diarrheal excretion, is recorded. The diarrhea-free period is defined as the time in minutes between castor oil administration and the occurrence of the first diarrheal output. The acute diarrheal

phase is the time between the first and the last diarrheal output of the 8-h observation period. Stools occurring between 8 and 24 h after castor oil administration are called late diarrheal excretion.

EVALUATION

With antidiarrheal agents dose-response curves are obtained for decrease of hypersecretion (stool weight) and for increase of the diarrhea-free period are obtained. Inhibitors of prostaglandin biosynthesis increase the diarrhea free period but do not affect early diarrheal secretion (Niemegeers et al. 1984).

MODIFICATIONS OF THE METHOD

Inhibition of castor oil-induced diarrhea in **mice** was tested by Bianchi and Goi (1977).

Dajani et al. (1977) tested antidiarrheal activity in castor-oil treated **monkeys**.

Mannitol-induced diarrhea was used as model in **calves** (Fioramonti and Buéno 1977) and in **pigs** (Théodorou et al. 1991).

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J.4.2.2 Antidiarrheal effect in cecectomized rats

PURPOSE AND RATIONALE

Evaluation of antisecretory antidiarrheal agents in animal models is limited primarily to extrapolations of efficacy from enteropooling studies *in vivo* (DiJoseph et al. 1984), isolated intestinal loops (Nakaki et al. 1982), and Ussing flux chamber preparations *in vitro* (Dharmsathaphorn et al. 1984).These studies do not mimic secretory diarrhea. The method of Magnus (1915) using chronic diarrhea in cats induced by continuous milk diet and thereby proofing the antidiarrheal effect of morphine is of historical interest only.

Fondacaro et al. (1990) developed a model of secretory diarrhea utilizing conscious cecectomized rats by surgical resection of the cecum and by use of potent intestinal secretagogues. The rat has a pronounced cecum as part of its gastrointestinal tract. The rat cecum is not only a reservoir for intestinal contents where high concentrations of various microbial flora assist in the digestion of carbohydrates, cellulose and peptides through microbial fermentation processes (Ambuhl et al. 1979; Williams and Senior 1982) but also plays a role in handling of excess intestinal fluid.

PROCEDURE

Cecectomies are performed in unfasted rats weighing 200–250 g. Under anesthesia with methohexital (60 mg/kg i.p.) cecectomy is initiated with a 2-cm midventral incision. The cecum is lifted from the abdominal cavity and exteriorized onto a gauze drape. The cecal apex is freed by severing the avasclular area of the mesocecum. A ligature of no. 1 silk suture is positioned so as to occlude the cecum and its vasculature without compromising ileo-colonic patency. After the ligature is secured and ileo-colonic patency confirmed, the cecum is resected, and the remaining ex-

posed cecal mucosa is washed with saline and cauterized. The intestinal segment is then returned to the abdominal cavity and the abdominal muscle fascia closed with sutures. The dermal incision is closed with wound clips that are removed about 1 week postsurgery. Immediately following the surgical procedure, the animals are returned to their cages and allowed free access to food and water. The animals are permitted at least a 48-h recovery period before being used in an experiment.

For the diarrhea assay, cecectomized rats are put into individual wire-bottomed cages placed over sheets of clean paper, and deprived of food and water for the duration of the assay. Rats are given a two hour's acclimatization period prior to the start of the assay in order to eliminate sporadic episodes of anxiety-induced defecation. During this period, they are observed also for consistent occurrences of pelleted feces; an animal producing other than pelleted stool is disqualified from the study. Diarrhea is induced with oral administration of secretagogues: either 16,16 dimethyl prostaglandin E_2 (0.3 mg/kg) in 3.5% ethanol, carbachol (15 mg/kg) in water, or cholera toxin (0.5 mg/kg) in an aqueous vehicle of 2% $NaHCO_3$ plus 2% casamino acids. Antidiarrheal agents are administered by gavage after the onset of diarrheal episodes. The cage papers are removed and examined at 15 min intervals for carbachol-induced diarrhea, 30 min intervals for 16,16 dimethyl prostaglandin E_2 induced diarrhea, and hourly when cholera toxin is used as secretagogue. Fetal output is recorded at each interval and scored as follows:

- 1 = normal pelleted stool
- 2 = soft-formed stools
- 3 = water stool and/or diarrhea

Known antidiarrheal agents, such as chlorpromazine (10 mg/kg p.o.), or the alpha-2 receptor agonist clonidine (1.0 mg/kg p.o.), or morphine (10 mg/kg p.o.) reduce the fecal output and induce a cessation of diarrhea.

EVALUATION

The fecal output index is defined as the summation of the number of defecation periods and their ranked consistency score within an observation period and is expressed as mean ±SEM for each group. Student's *t*-test and analysis of variance are used for statistical comparisons of data points. Significance is accepted at $p < 0.05$ or less.

CRITICAL ASSESSMENT OF THE METHOD

The model of diarrhea induced by secretagogues in cecetomized rats has the advantage to mimic secretory diarrhea in man.

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J.4.2.3 Evaluation of antidiarrheal effect in cold-restrained rats

PURPOSE AND RATIONALE

Barone et al. (1990) tested the effect of various antidiarrheal and other drugs on increased fecal pellet output in cold-restrained rats resembling clinical observations that stressful situations can produce diarrhea in humans.

PROCEDURE

Male Sprague-Dawley rats weighing 260–310 g are maintained on Purina lab chow and water. Since gastric ulcers are reduced if cold-restrained rats are allowed free access to food and water, for studies on fecal output food was not withdrawn prior to the experiment. The rats are studied in normal living cages at room temperature (control, non-stressed animals) or in wire-mesh restraining cylinders placed in a cold (4 °C) environment. Test drugs are administered by appropriate routes over optimal effective dose-ranges for their activities and at optimal pretreatment times to maximize their effects. The number of pellets expelled by each animal is measured at 1 and 3 h (fecal pellet output). Generally, the fecal pellets of stressed animals are less firm.

Fecal pellet fluid content is determined by weighing fecal pellets, drying them in an oven at 37 °C, and weighing them again.

EVALUATION

The dose in mg/kg that inhibits the cold restrained stress induced increase in fecal pellet output by 50% (ID_{50}) is determined using least-squares fit analysis directly from the regression line. If fecal pellet output is decreased by a drug but no clear dose-related effects occur, the maximum percent decrease is determined.

MODIFICATIONS OF THE METHOD

For colonic transit studies, rats are implanted with indwelling catheters in the proximal colon. Animals are anesthetized with 60 mg/kg pentobarbital i.p., and a chronic colonic catheter is positioned to enter the proximal colon 2 cm from the ileocecal junction. A catheter of about 20 cm of silicone tubing prepared with several drops of silicon rubber adhesive coating a 1-cm length along the catheter is positioned into the colon. The colon end of the tubing also is sealed with a 1-cm plug of petroleum jelly intraluminally. A small incision is made in the proximal colon, and the adhesive-coated portion of the Silastic tubing is tied in place with the use of a purse-string suture. The tubing is brought through the abdominal wall, led subcutaneously through the skin in the midscapular region and secured on the back of the neck with the use of a wound clip. The abdominal incision is closed with sutures and wound clips.

Experiments are performed in conscious animals 48–72 h after surgical preparation. At this time, the radiolabeled marker (^{51}Cr as sodium chromate) is instilled into the proximal colon via the indwelling catheter. After 35, 60, or 120 min, cold-restrained rats and controls are sacrificed and their colons and large intestines are removed. The cecum and equal segments of the colon are dissected, placed into vials and subjected to gamma counting.

Ikeda et al. (1995) investigated the effect of a neurokinin$_1$ receptor antagonist on stress-induced defecation in rats placed in special restraint cages.

Kishibayashi et al. (1993) studied distal colonic function using wrap-restrained stress-induced defecation as described by Williams et al. (1988). The rats were lightly anesthetized with ether, and the foreshoulders, upper forelimbs and the thoracic trunk were wrapped in paper tape to restrict, but not prevent, movement. The animals recovered from anesthesia within 2–5 min and immediately moved around in cages and ate and drank, but had been restricted from mobility of forelimbs, which prevented them from grooming the face, upper head and the neck. Fecal pellet output induced by wrap-restraint stress was weighed during the 1st h after stress. The test drugs were given p.o. 1 h before stress. The ID_{50} values were calculated as the doses that reduced stress-induced defecation by 50%.

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J.4.3 Gut motility

J.4.3.1 Isolated ileum (MAGNUS technique)

PURPOSE AND RATIONALE

The isolated ileum, as first described by Magnus (1904), is probably the most widely used model in experimental pharmacology. Magnus already studied simultaneously the spontaneous contractions of the longitudinal and circular musculature and the inhibiting effect of atropine. The method has been used for many purposes, such as the study on the effects of adrenaline on the lower segments causing contraction and on the segments of the upper end causing relaxation by Munro (1951) or the study on the origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips by Paton and Zar (1968) either retaining or being denervated from Auerbach's plexus. The model is used as a basic screening procedure for spasmolytic activity, whereby an anti-acetylcholine or anticarbachol effect indicates antimuscarinic activity and an anti-$BaCl_2$-effect indicates a musculotropic, papaverine-like effect. In addition to the isolated ileum, other parts of the gut such as the isolated duodenum and colon, have been used widely.

PROCEDURE

Guinea pigs of either sex weighing 300 to 500 g are used. They are sacrificed by stunning and exsanguination. The abdomen is opened with scissors. Just distal to the pylorus, a cord is tied around the intestine which is then severed above the cord. The intestine is gradually removed, with the mesentery being cut away as nec-

essary. When the colon is reached, the intestine is cut. Below the cord, the intestine is cut halfway through, so that a glass tube can be inserted. Tyrode's solution is passed through the tube and the intestine until the effluent is clear. Mesentery is cut away from the intestine that was joined to the colon. Pieces of 2–3 cm length are cut. Preferable the most distal piece is used being the most sensitive one. This piece is fixed with a tissue clamp and brought into a 15 ml organ bath containing Tyrode's solution at 37 °C being oxygenated with 95% O_2/5% CO_2. The other end is fixed to an isometric force transducer (UC 2 Gould-Statham, Oxnard USA). A preload of 1 g is chosen. Responses are recorded on a polygraph. After a pre-incubation time of 30 min, the experiment is started.

The following agonists and antagonists (standards) are used (concentrations in g/ml bath fluid):

Agonist		Antagonist	
Acetylcholine	10^{-7} g/ml	Atropine	10^{-8}–19^{-9} g/ml
		Scopolamine	10^{-8}–10^{-9} g/ml
Carbachol	10^{-7} g/ml	Atropine	10^{-8}–10^{-9} g/ml
Histamine	10^{-6} g/ml	Histamine antagonists	
$BaCl_2$	10^{-4} g/ml	Papaverine	10^{-5}–10^{-6} g/ml
Serotonin	10^{-6} g/ml	Serotonin antagonists	
PGE_2	2×10^{-7} g/ml	PG-antagonists	

EVALUATION

Several methods for the quantitative evaluation of an antagonistic effect are available. One approach is the determination of pD_2' values according to van Rossum and van den Brink (1963). Acetylcholine or histamine is added in 1/2 $\log_{10}$ concentration increments until a maximum response is obtained. Control curves are recorded at 30 min intervals. After uniform control responses are obtained, the potential antagonist or the standard is added 5 min before the concentration-response curve is re-obtained. The potency of the antagonist is obtained by calculating the pD_2' value which is defined as the negative logarithm of the molar concentration of an antagonist that causes a 50% reduction or the maximal response obtained with an agonist.

MODIFICATIONS OF THE METHOD

Many modifications of the Magnus technique have been described in the literature, mainly with the isolated ileum (e.g., Koelle et al. 1950).

Okwuasaba and Cook (1980) dissected the myenteric plexus and longitudinal muscle free of the underlying circular muscle according to the method of Paton (1957), Paton and Zar (1968) and stimulated the preparation with trains of supramaximal rectangular pulses of 1.0 ms duration at a frequency of 0.2 Hz.

Kilbinger et al. (1995) studied the influence of 5-HT_4 receptors on [^{3}H]-acetylcholine release from guinea pig myenteric plexus.

De Graaf et al. (1983) described a fully automated system for *in vitro* experiments with isolated tissues. The apparatus consists of an organ bath equipped with (a) a gradient pump supplying a logarithmic concentration/time gradient of agonist; (b) pumps and valves for dispensing bath fluid, antagonist solutions, and an oxygenation gas mixture; and (c) a transducer with automatic baseline adjustment. The information coming from the preparation is fed into a mini-computer. The data of various experiments can be accumulated and Schild-plots obtained.

Furukuwa et al. (1980) studied the effects of thyrotropin-releasing hormone on the isolated small intestine and taenia coli of the guinea pig.

Paiva et al. (1988) studied the role of sodium ions in angiotensin tachyphylaxis in the guinea-pig ileum and taenia coli.

Barnette et al. (1990) used electrically stimulated strips of circular smooth muscle from the lower esophageal sphincter of dogs to study the inhibition of neuronally induced relaxation by opioid peptides.

Bradykinin antagonism can be studied in the isolated guinea pig ileum bathed in a solution containing atropine (1.5 mM), diphenhydramine (3.4 mM), indomethacin (2.8 mM), and captopril (0.9 mM) (Rubin et al. 1978; Kachur et al. 1987).

Griesbacher and Lembeck (1992) used the isolated guinea-pig ileum for analysis of bradykinin antagonists.

Hew et al. (1990) used field stimulated (95% of maximum voltage, 0.1 Hz, 0.5 ms) guinea pig ileum, bathed in physiological salt solution at 37 °C in the presence of 1 mM mepyramine for determination of histamine H_3 bioresponse. Reduction of contractile response by the test substance (>50% relative to control 0.3 mM R-α-methylhistamine) indicates possible H_3 agonism. At a test concentration where no significant activity is seen, ability to inhibit (>50%) R-α-methylhistamine-induced contractile reduction indicates antagonistic activity.

A similar technique was used by Conner et al. (1987) to study antagonist effects at functional 5-HT_{1A} receptors.

Feniuk et al. (1993) used the guinea-pig isolated ileum, vas deferens and right atrium to characterize somatostatin receptors. Transmural electrical stimulation was applied to guinea pig ileum (0.1 Hz, 0.1 ms continuously) and vas deferens (5 Hz, 0.5 ms for 1.5 s every 30 s) at supramaximal currents (approximately 800 mA) delivered from a Digitimer D330 multistimulator.

Radomirow et al. (1994) investigated opioid effects of short enkephalin fragments containing the Gly-Phe sequence on contractile responses of guinea pig ileum after addition of 10 nM acetylcholine or after electrical stimulation.

Coupar and Liu (1996) described a simple method for measuring the effects of drugs on intestinal longitudinal and circular muscle in **rats**. The preparation consists of a segment of rat ileum set up to measure the tension developed in the longitudinal muscle and intraluminal pressure developed in the circular muscle in response to transmural electrical stimulation.

Vassilev et al. (1993) exposed Wistar rats to subtoxic doses of Co^{2+} or Ni^{2+}, receiving $Co(NO_3)_2$ or $NiSO_4$ with drinking water for 30 days, and measured the changes in the contractile responses to carbachol and in the inhibitory effects of verapamil and nitrendipine on isolated smooth muscle preparations of the ileum and the trachea.

Pencheva and Radomirov (1993), Pencheva et al. (1999) studied the effects of GABA receptor agonists on the spontaneous activity of the circular layer in the terminal ileum of **cats**. Segments of the terminal ileum approximately 0.5 cm long were mounted in an organ bath along the axis of the circular layer through a cotton thread with a large knot situated at the inner part of the gut wall.

Similar preparations of cat ileum were used by Kortezova et al. (1994) and Chernaeva and Mizhorkova (1995).

Vassilev and Radomirov (1992) used an isolated preparation of **rat rectum**. The rectal region, 1–6 cm proximal to the anal sphincter was removed and a 20 mm long segment suspended in an organ bath. The influence of prostaglandins and antagonists on spontaneous mechanical activity and electrically stimulated responses was investigated.

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J.4.3.2 Cascade superfusion technique

PURPOSE AND RATIONALE

The technique of isolated organ superfusion was developed by Gaddum (1953) for the assay of biologically active substances. Extension of the technique for multiple tissue superfusion with particular reference to the identification and the assay of prostaglandin-like activity was used by various authors (Vane 1964; Ferreira and Vane 1967; Ferreira et al. 1976; Gilmore et al. 1968; Hong 1974; Bult et al. 1977; Henman et al. 1978; Elliott and Adolfs 1984; Fournau et al. 1984).

PROCEDURE

The apparatus consists of a double-wall glass container (height 20–25 cm, inner diameter 7–8 cm) with an outlet at the bottom. A constant temperature of 38 °C is maintained by circulation of warm water through the outer jacket. Inside the glass container can be suspended up to 5 pieces of tissue of various origins. The multiple preparation tissue holder consists of a vertical rectangular rod and plastic platforms for attachment of the tissues and for accurate deflection of the superfusate on to the lower tissue. The rod is grooved at 10 mm intervals with 1 mm deep slots set at an angle of 20° to the horizontal. To the upper surface of the nonwettable platform a small plastic hook is cemented at such a distance from the rod that when the tissue is in position its attachment thread passes between the V-shaped notch cut into the margin of the upper tissue platform. The individual platforms are inserted on to the vertical rod by slotting into the requisite grooves appropriate to the tissue length.

Thus, the superfusate passes at a uniform flow rate down the tissues of the cascade and the tension recording threads are separated from each other by about a 5-mm gap so that the responses can be conveniently recorded. The threads from the organs are connected over isotonic levers to isometric tension transducers. The lever is used for preloads according to the individual organ. Tension exerted by each tissue is recorded on a polygraph. Various media can be used for superfusion, e.g., Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2.

Many tissue preparations can be used for the cascade, such as rat fundic strip, rat duodenum, rat colon, rat bladder strip, guinea-pig ileum, guinea-pig proximal colon, rabbit stomach strip, rabbit coeliac/mesenteric artery, or rabbit aorta strip. Moreover, donor tissue can be superfused and its effluent be tested in the organs of the cascade.

EVALUATION

Many agonists and antagonists can be tested by appropriate selection of organs. Threshold doses and ED_{50} or ID_{50} values can be determined.

MODIFICATIONS OF THE METHOD

The superfusion technique has been used for several purposes, e.g., for the assay of catecholamines (Armitage and Vane 1964), for detecting active substances in the circulating blood (Vane 1964). A simple and inexpensive piece of apparatus for cascade superfusion procedures has been described by Naylor (1977).

Mombouli et al. (1996) described a bioassay of endothelium-derived hyperpolarizing factor (EDHF) using a perfusion-superfusion cascade where canine carotid arteries were used as donors of vasoactive substances and rings of coronary arteries without endothelium as detectors.

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J.4.3.3 *In vivo* evaluation of spasmolytic activity in rats

PURPOSE AND RATIONALE

Maggi and Meli (1982) described an *in vivo* procedure for estimating spasmolytic activity in the rat by measuring smooth muscle contractions to topically applied acetylcholine.

PROCEDURE

Male albino rats weighing 350–400 g are anesthetized with subcutaneous urethane (1.2 g/kg). The left jugular vein is cannulated for administration of test compounds. After laparatomy occluding silk ligatures are applied at a distance of 2 cm from each other in the gut (colon or rectum). Through a small incision the flanged tip of a polyethylene tubing (1 mm i.d., 1.5 mm o.d.) is inserted into the lumen of this pocket-like space and secured in place by a purse string ligature. The free end of the tubing is connected to a pressure transducer and the whole system filled with saline. The same procedure is performed with the urinary bladder. The organs are filled with warm saline (37 °C) to obtain a resting pressure of 4–12 mm Hg. Warm saline soaked cotton wool swabs are laid around the exteriorized organs which are maintained warm and moist with warm (37 °C) saline dropping from a reservoir at a rate of 10–15 drops/min.

After 15 min stabilization period, saline flow is stopped and a dose-response curve to acetylcholine determined. A volume of 0.5 ml (an amount sufficient to put the whole outer surface of the organ into contact with the bathing solution) of acetylcholine at the desired concentration is applied within 2–3 s from a syringe to the outer surface of the target organ. To construct a dose-response curve of acetylcholine, increasing concentrations are applied to the target organ until maximal contraction is obtained. After at least 3 or more control curves have been obtained at 10 min intervals, the antagonist is administered.

EVALUATION

The quantitative analysis of the data is carried out by plotting the results of each experiment as log (acetylcholine dose ratio-1) against log dose antagonist (Arunlakshana and Schild 1959). The regression line is calculated according to the method of least squares and ED_{50} values and 95% confidence limits according to Litchfield and Wilcoxon (1949). From ED_{50} values, the dose of the antagonist (mg/kg i.v.) to produce an acetylcholine dose ratio of 10 is calculated according to Daly et al. (1975). Parallel displacement to the right of the agonist dose response curve and a slope of unity for the regression line indicate competitive antagonism. The DR_{10} values from different organs are compared by means of Student's *t*-test for unpaired data.

MODIFICATIONS OF THE METHOD

In vivo registration of gut motility in guinea pigs was already described by Straub and Viaud (1933). A four cm long part of the gut was ligated and filled with Tyrode solution. Gut motility was measured at variable intraluminal pressure.

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J.4.3.4 Colon motility in anesthetized rats

PURPOSE AND RATIONALE

The influence of spasmolytic drugs on carbachol induced increase of colonic motility can be measured in anesthetized rats. The method has also be used to study the stimulation of colonic motility by an enkephalin analogue pentapeptide (Bickel 1983).

PROCEDURE

Male Sprague-Dawley rats weighing 350–500 g are anesthetized with pentobarbital i.v. A pressure sensitive tip catheter is inserted into the colon ascendens and the signals of the intraluminal pressure changes

are recorded. The colonic contractions are stimulated by i.v. injection of 3 mg/kg carbachol. The height and the duration of the contractions are recorded. Then, the test compound is injected intravenously. The decrease of contractions is measured and the duration of the spasmolytic activity determined by repeated administration of carbachol at 15 min intervals, until the contractions are not significantly different from the response obtained with carbachol alone.

EVALUATION

Significant differences are calculated using Student's unpaired *t*-test.

MODIFICATIONS OF THE METHOD

Maggi and Meli (1984) used eserine-induced hypertonus of **guinea pig** distal colon *in vivo* as a pharmacological procedure for testing smooth muscle relaxants. Male albino guinea pigs weighing 240–300 g are anesthetized with 1.5 g/kg urethane s.c. Through a midline abdominal incision, the proximal part of the hypogastric loop of the distal colon is exposed and occluding silk ligatures are applied at a distance of 2 cm from each other, taking great care to avoid any lesion to the vascu-lar and nervous supply. Through a small incision, the flanged tip of a polyethylene tube (1 mm i.d., 1.5 mm o.d.) is inserted into the lumen and secured by means of a purse-string ligature. The free end of the tube is con-nected to a pressure transducer and the whole system is filled with saline. Intraluminal pressure and its variations are recorded on a polygraph. The effect of drugs is assessed as inhibition of eserine induced hypertonus.

Théodorou et al. (1991) studied the absorptive and motor components of the antidiarrheal action of loperamide in **pigs**. Motility was recorded by implantation of intraparietal electrodes into various parts of the gut.

Raffa et al. (1987) used a method utilizing the insertion of a 3 mm glass bead into the distal colon in **mice** to evaluate the activity of intracerebroventricularly administered μ- and δ-opioid agonists on colonic bead expulsion time.

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J.4.3.5 Continuous recording of electrical and mechanical activity in the gut of the conscious rat

PURPOSE AND RATIONALE

Bueno et al. (1981) described a method for continuous electrical and mechanical activity recording in the gut of the conscious rat. In this study two methods for the continuous recording of motor events – microtransducers and the electromyogram – are compared for the rat stomach and intestine.

PROCEDURE

Male Wistar rats weighing 200–300 g are housed singly in wire-bottomed cages and allowed lab chow and water ad libitum during the training period of 30 days. Under halothane anesthesia pairs of electrodes are implanted along the greater curvature of the pyloric antrum 5 and 2 cm from the pylorus, on the antimesenteric border of the duodenum 2 and 5 cm beyond the pylorus, and along the jejunum, 4 cm from the pylorus using a procedure described by Ruckebusch and Fioramonti (1975).

The contractions of the circular muscle layer are recorded with strain gauge microtransducers sutured onto the serosa at less than 1 cm from each set of electrodes (Pascaud et al. 1978). The free ends of the electrodes and strain gauge wires are carried subcutaneously and exteriorized on the back of the neck. The wires are inserted into a glass tube (15 cm and 0.5 cm external diameter) to prevent twisting and any contact with the metallic lids of the cage.

EVALUATION

Both electrical and mechanical activities are continuously recorded starting 5–7 days after surgery on a multichannel recorder (e.g., 8-channel Dynograph®, Beckman, USA) using lead selector couplers (type 9 856, Beckman) at a constant time of 0.1 s for the electrical spiking activity and strain gauge couplers (type 9 863, Beckman) for the contractile force minitransducers. Simultaneously low frequency signals (frequency < 3 Hz) of the electromyogram are eliminated through filters and the spiking activity, integrated for each 20 s, is recorded on a potentiometric recorder. The electrical and

mechanical activities are also recorded simultaneously on a magnetic tape recorder.

The index of motility, expressed as mcoul/min is calculated from the integrated records of electrical activity. The index of mechanical motility is calculated as the area under all contraction waves occurring during 1 min, and is expressed as g s/min. Mechanical and electrical activities during a test period of 30 min after administration of stimulant drugs, e.g., the gastrointestinal hormones gastrin and cholecystokinin, or relaxing drugs, like anticholinergic agents, are compared with the values of a 30 min pretest period.

MODIFICATIONS OF THE METHOD

Wright et al. (1981) described a similar method for long-term recording of intestinal mechanical and electrical activity in the unrestrained rat. Mechanical activity is detected using miniaturized half-bridge metal foil strain-gauge force transducers. The electrical activity is monitored by silver/silver chloride bipolar electrodes. The lead wires from the recording units are encased in a metal compression spring and are permanently joint to a ball connector positioned on the top of the cage, such allowing the animal free access to all parts of the cage.

Stam et al. (1995) described computer analysis of the migrating motility complex of the small intestine recorded in freely moving rats. Myoelectric activity of the small intestine was recorded digitally in fasted, freely moving rats with multiple pairs of electrodes in the antimesenterial smooth muscle. A computer program was developed to distinguish the three characteristic phases of the migrating motility complex.

Fändriks (1993) measured duodenal wall motility, mucosal fluid transport and alkaline secretion in anesthetized **cats**. A triple-lumen tube supplied with two small balloons was positioned via the esophagus in the duodenal lumen and its distal end was led through a small incision in the antimesenteric border of the distal duodenum at the level of the ligament of Treitz. The most oral of the balloons was positioned immediately to the pylorus. After filling with air, the balloons occluded the lumen and isolated a 2 cm segment of the proximal duodenum. A double-lumen tube was inserted into the stomach for luminal perfusion.

Martinez et al. (1993), Jimenez et al. (1994) studied gastrointestinal motility and coordination in **chickens**. Animals were chronically implanted with electrodes in stomach, duodenum, ileum, caeca and rectum.

Nakajima et al. (1996) used a telemetric device (supplied by Data Sciences International, Inc., St Paul, MN) which can be implanted in the abdominal cavity of small animals. Gastric motility of freely moving rats could be continuously recorded for up to 60 days.

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J.4.3.6 Propulsive gut motility in mice

PURPOSE AND RATIONALE

The passage of a charcoal meal through the gastrointestinal tract in mice is used as parameter for intestinal motility and to study the effect of laxatives.

PROCEDURE

Groups of 10 female mice (e.g., NMRI strain) weighing 15 g are fed an oat diet for 3 days. Eighteen hours prior to the experiment food, but not water, is withdrawn. The animals are treated either subcutaneously 15 min or orally 60 min before administration of the charcoal meal (0.2 ml of a 4% suspension of charcoal in 2% carboxymethylcellulose solution). The mice are sacrificed after various time intervals, 20 min, 40 min, 60 min and 120 min. Ten animals serve as controls for each time interval. The entire intestine is immediately removed and immersed in 5% formalin to halt peristalsis; then washed in running water. The distance the

meal has traveled through the intestine as indicated by the charcoal is measured and expressed as percent of the total distance from the pylorus to the caecum.

EVALUATION

Student's *t*-test is used to compare the control and the drug-treated group.

CRITICAL ASSESSMENT OF THE METHOD

The charcoal passage test can be used for evaluation of laxative activity as well as for inhibition of intestinal motility.

MODIFICATIONS OF THE METHOD

Instead of charcoal, unsubstituted Hostapermblau (CuPcB) suspended in gummi arabicum mucilage can be used.

Carmine red (15) suspended in a 1% tragacanth solution was used for measurement of small intestine transit in **rats** (Leng-Peschlow 1986).

Miller et al. (1981) measured the intestinal transit in the rat by the use of radiochromium (^{51}Cr). Female Sprague Dawley rats weighing approximately 200 g were implanted with indwelling silastic cannulae in the proximal duodenum. Following a 3 day recovery period, the animals were fasted for 18 h and then treated with the test compounds. Thirty min later, 0.2 ml of radiochromium (0.5 mCi $Na^{51}CrO_4$) was instilled into the small intestine via the indwelling silastic cannula. Twenty-five min after chromium instillation, the animals were sacrificed. The small intestine was carefully removed and divided into 10 equal segments. The radioactivity was determined with an automatic gamma counting system. The effect of drugs could be quantified by determining the geometric center of the distribution of chromium through the small intestine.

Shook et al. (1989) used radiolabeled chromium to measure gastrointestinal transit in mice.

Megens et al. (1989) used the charcoal test to study the *in vivo* dissociation between the antipropulsive and antidiarrheal properties of opioids in rats.

Lish and Peters (1957) recommended an intestinal antipropulsive test in intact insulin-treated rats providing certain advantages over the commonly used charcoal meal test for screening of synthetic antispasmodic and antipropulsive agents.

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J.4.3.7 Nerve-jejunum preparation of the rabbit

PURPOSE AND RATIONALE

Perivascular nerve stimulation induces cessation of peristalsis of rabbit jejunum (Finkelman 1930). Effects of test compounds on this phenomenon can be tested.

PROCEDURE

Albino rabbits are sacrificed and the jejunal part of the gut prepared. The nerve lies in the mesentery along with the arterial blood supply. Nerve-jejunum preparations are suspended in an organ bath. The preparation is stimulated with pulses at a frequency of 20 Hz with 0.5 ms and about 5 volt for 10 s at 3 min intervals. Test compounds are applied to the organ bath in a cumulative manner at 6 min intervals. Peristalsis movements for each period of 3 min between drug application or of the period of cessation of peristalsis induced by nerve stimulation of the rabbit jejunum preparation are recorded. The effect of drugs on spontaneous peristalsis movement and on the cessation of peristalsis movement exerted by perivascular nerve stimulation is tested.

EVALUATION

The areas of peristalsis movement for each period of 3 min are measured using a planimeter. The effects are expressed as percentage of change between controls and test compound treated preparations.

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J.4.3.8 Motility of gastrointestinal tract in dogs

PURPOSE AND RATIONALE

Intraluminal pressure and motility of the small intestine can be measured in unanesthetized dogs with balloon catheter systems via a duodenal Mann-Bollman (1931) fistula according to Tasaka and Farrar (1976) or in the loop of a Thiry-Vella fistula (see below).

PROCEDURE

Fistulas of the small intestine are created in male Beagle dogs weighing 15–20 kg. The animals are anesthetized with 40 mg/kg pentobarbital i.v. and fixed on an operation table. After shaving and careful disinfection of the skin a midline incision is made. A 10–15 cm length of ileum, approximately 15 cm proximal to the caecum, is excised. The remaining ileum is anastomosed end-to-end. The excised ileum is anastomosed, end to side, to the proximal or middle jejunum. Radiopaque tantalum markers are sutured to the serosa distal to this anastomosis in order to guide the direction for subsequent intubation. The other end is sutured to the skin. To create a skin ileostomy which does not shrink rapidly, a small amount of muscle, fascia and subcutaneous tissue are excised from the abdominal wall.

For the measurement of the pressure inside the intestine, an air-filled system is employed. Air-filled latex balloons (Cementex), 5 mm in diameter, are attached to air-filled PE 190 polyethylene catheters (ID 1.19 mm) with a length of 120 cm. Three balloon-catheter pressure assemblies are tied together with the balloons 5 cm apart. The catheters are connected to Statham p23 db pressure transducers and to a polygraph. The transducers are rendered airtight by repeated applications of latex to all the connections.

The dogs are withheld from food, but not from water 18 h prior to the experiment. The balloon-catheter assemblies are introduced through the fistula and secured in an appropriate position. The system is filled with air to a pressure of 10 mm Hg. Similarly, balloon-catheter assemblies can be introduced into a Thiry-Vella fistula.

Intraluminal pressure is measured continuously; frequency and amplitude of pressure waves are recorded. After a period of 1 h, the test drug is administered orally or subcutaneously and the above mentioned parameters recorded for 10 min intervals.

EVALUATION

For 10 min intervals amplitude frequency (f_A), average degree and duration of amplitudes (A) and average pressure performance (PL_i) are calculated. Post-drug values are compared with pre-drug readings.

MODIFICATIONS OF THE METHOD

Goldenberg and Burns (1973) reported on a technique using rubber balloon catheters inserted in the duodenum, ileum, or colon of dogs, secured by purse-string sutures and filled with water to record intraluminal pressure monitored by Statham pressure transducers connected to a polygraph. Furthermore, an antispasmodic agent can be tested for relaxation of morphine sulfate (0.3 mg/kg i.v.) induced spasms of the intestinal tract.

Fox et al. (1985) implanted chronically extraluminal strain gage force transducers on the serosal surface of the gastrointestinal tract of dogs.

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J.4.3.9 Thiry-Vella fistula

PURPOSE AND RATIONALE

As described first by Thiry (1864) and improved by Vella (1892), a part of the jejunum is isolated and the ends are exteriorized through the abdominal wall allowing to measure *in vivo* motility and function of the intestines in dogs and other species (Boldyreff 1925).

PROCEDURE

Male Beagle dogs weighing 15–20 kg are used. They are fasted 24 h preoperatively. Intravenous pentobarbital sodium, 30 mg/kg, provides satisfactory anesthesia. It can be supplemented, if necessary, during operation. The abdominal part is shaved with electric clippers, then with a razor. The skin is disinfected with Zephiran® –70% alcohol. Sterile drapes are applied to cover the whole operative field. A mid-line linea alba incision is made. A loop of the jejunum, about 70 cm in length, is separated leaving the blood supply through the mesenterium intact. Both distal and proximal ends are exteriorized through the abdominal wall and provided with stomata. An end-to-end jejuno-jejunal anastomosis is performed.

EVALUATION

The preparation can be used to evaluate intestinal motility. A latex balloon connected via a polyethylene catheter to a pressure transducer (Statham P 23 BB) is introduced through the proximal ostium. Changes in intragastric pressure are measured on a frequency measurement bridge and are recorded continuously. The number and height of the pressure waves are used as indices of intestinal motor activity. Secretin inhibits motility of the small intestine dose-dependent.

MODIFICATIONS OF THE METHOD

Sarr et al. (1981) and Bastidas et al. (1990) used **dogs** with Thiry-Vella loops to study jejunal absorption.

Bilchik et al. (1993) examined the effects of physiological doses of peptide YY in dogs with jejunal and ileal exteriorized, neurovascularly intact Thiry-Vella fistulas.

Ashton et al. (1996) created Thiry-Vella fistulas using a 20 cm segment of distal colon under general anesthesia in dogs. Colonic absorption of water and electrolytes was evaluated in awake, conscious animals.

Liu et al. (1996a) studied the effects of intravenous peptide YY on colonic water and electrolyte transport in awake dogs which had 20 cm neurovascularly intact colon Thiry-Vella fistulas.

In an other study, Liu et al. (1996b) evaluated the effects of cholecystokinin and peptide YY on intestinal absorption of water and electrolytes using colonic, ileal, or jejunal fistulas in dogs.

These authors (Liu et al. 1995) also examined the effects of intraluminal administration of a new substituted peptide YY analog on intestinal absorption of water and electrolytes in dog with jejunal, ileal and colonic Thiry-Vella fistulas.

Barry et al. (1995) investigated the effect of luminally administered dopamine, the D_1-receptor agonist SKF 38 393, the α_1-receptor antagonist terazosin, and the α_2-receptor antagonist yohimbine on ileal water and electrolyte transport in dogs with Thiry-Vella fistulas.

Walters et al. (1994) described the effect of a model of canine jejuno-ileal orthotopic autotransplantation on jejunal and ileal transport of water and electrolytes. For neurally intact jejunal loops, myoneural continuity between the loop and the proximal duodenum and jejunum was maintained by preserving a bridge of tunica muscularis devoid of mucosa between the proximal jejunum and the loop. All intrinsic neural and lymphatic continuity to the loop was carefully maintained by not transecting any of the mesentery to the loop.

For the autotransplanted jejunal loop the dogs underwent construction of an identical loop with the muscular bridge after preparation of an jejuno-ileal autotransplantation. For this purpose, all neural, lymphatic, myogenous, and connective tissue connections with the jejuno-ileum were transected except for the fully isolated and stripped superior mesentery artery and vein at the base of the small bowel mesentery. The perivascular and adventitial tissue of these two vessels were carefully dissected away and transected under optical magnification. From here, the mesenteries to the distal duodenum and to the distal ileum were transected in radial fashion and the distal duodenum and distal ileum 5 cm from the ileo-colonic junction were also transected. At this point, the jejuno-ileum was completely isolated from any neural/lymphatic continuity with the dog except for any neural/lymphatic elements travelling within the media of the mesenteric artery and vein.

Neurally intact and autotransplanted ileal loops were prepared in a similar way.

Remie et al. (1990) described in detail the preparation of a Thiry-Vella loop in the **rat**. After laparatomy the segment to be isolated has to be located carefully. The segment should be vascularized by two or more tributary arteries. For ligation of the arcade, two ligatures are placed around the blood vessels 2–3 mm from each other. The two ligatures are tightened, the gut wall is disinfected with iodine solution, and both the vessels of the arcade and the gut wall are cut. The same procedure is performed at the other end of the segment to be isolated. This segment is laid on gauze moistened with warm saline avoiding torsion of the vessels. For end-to-end anastomosis the remaining parts of the gut are approximated and two corner sutures are placed. A continuous suture with transfixing stitches is placed on the anterior and posterior wall. Using a pair of sharp scissors two holes are made in the abdominal wall and the skin. Subsequently, standard end-to-side anastomosis technique is used to sew the gut to the internal abdominal muscle. The two stay sutures are at 180° and the posterior wall is sutured first. Following this, the skin is sutured to the abdominal muscle, using a running suture.

Chu et al. (1995) used Thiry-Vella fistulas of either the jejunum or ileum in rats in order to determine whether the trophic effects of bombesin on the small bowel mucosa are mediated by nonluminal factors or endogenous luminal secretion.

Bárdos and Nagy (1995) prepared double Thiry-Vella fistulas in rats. The first Thiry-Vella loop was created from the lower duodenum. After 1 month or more, a similar fistula was prepared from the upper part of the colon. The two loops were positioned along the midline and formed a line of 4 openings of the abdominal wall providing an easy access for inserting stimulatory devices to either of the loops in the same animal. A rubber balloon made of latex rubber was tied to a

silicon rubber tube, inserted into the isolated loop via one of its orifices and then fixed to the body by a tape. Stimulation was performed by injection of various volumes of water into the balloon from an attached syringe. Behavioral reactions of the animals were recorded as scores during 10 s stimulation periods.

In order to purify the putative luminal cholecystokinin-releasing factor, Spannagel et al. (1996) collected intestinal secretions by perfusing a modified Thiry-Vella fistula of jejunum in conscious rats.

Gianotti and Tchervenkov (1992) used a Thiry-Vella loop in **guinea pigs** to study the stimulatory effect of intraluminal nutriment on burned guinea pig intestinal mucosa.

Philpott et (1993) created Thiry-Vella loops in 21 week old **rabbits** in order to study the influence of luminal factors on intestinal repair during refeeding of malnourished infant rabbits.

Silbart et al. (1996) examined the ability of several bacterial endotoxins and their subunits to act as adjuvants or carrier proteins in stimulating an intestinal secretory Ig4 response to 2-acetylaminoflourene using Thiry-Vella loops in rabbits.

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J.4.3.10 Continuous recording of mechanical and electrical activity in the intestine of conscious dogs

PURPOSE AND RATIONALE

Cyclic motor activity occurring in almost all parts of the gastrointestinal tract is due to a migrating electric

complex (Szurszewski 1969; Fioramonti et al. 1980; Sarna and Condon 1984; Sarna et al. 1984; Sarna 1985). Smooth muscle cells of the gut show periodic oscillations of membrane potentials called electrically controlled activity; they are also named slow waves, basic electrical rhythm, or pacesetter potentials. If the membrane potential depolarizes beyond a certain threshold during such an oscillation, the smooth muscle contracts. This is usually associated with a rapid burst of electrical oscillations, called electrical response activities or spikes. Electrical response activity is, therefore, associated with contractions on a 1 : 1 basis.

Implantation of extraluminal force transducers allows the monitoring of contractile activity in conscious animals (Bass and Wiley 1972; Ormsbee and Bass 1976; Ormsbee et al. 1981). The data can be analyzed by computer (Ehrlein and Hiesinger 1982; Schemann et al. 1985). The effects of opiate agonists, morphine, natural enkephalins, endorphins, and synthetic enkephalins and opiate antagonists, like nalorphine, as well as other gastrointestinal hormones, such as motilin, can be studied (Bickel and Belz 1985, 1988; Bickel et al. 1985).

PROCEDURE

Male Beagle dogs weighing 15–20 kg are anesthetized with thiobarbital and anesthesia is then maintained with halothane and a mixture of N_2O and O_2 (3 : 1). Under aseptic conditions several miniaturized strain gauge force transducers are sutured onto the muscular layers of the gastrointestinal tract. Before implantation, the transducers are externally calibrated giving linear signals over a range from 10 to 300 mN. Each transducer has its recording axis perpendicular to the longitudinal axis of the intestine to record contractions of the circular smooth muscles. The proximal ends of the strain gauge transducers are fitted to a plug embedded in a stainless-steel cannula implanted into the abdominal wall. The signals are continuously recorded and stored in a HP 9 835A Hewlett Packard computer, allowing later analysis of the data.

For measuring electrical activity, bipolar electrodes are implanted at several parts of the intestinal tract. The lead wires from the electrodes are externalized via a stainless-steel cannula. Electromyogram is recorded on an electroencephalograph.

The animals are kept in a standardized environment with one daily feeding at 9:00 A.M. The dogs are trained to stand in a Pawlov stand. Eighteen hours prior to the beginning of the experiment, the animals are fasted with access to water. Motility data and electrical activity are first recorded for fasting dogs without drug treatment. Then the drug is injected intravenously during the period of quiescence of the migrating motor complex.

EVALUATION

Recordings are analyzed for duration of cycles (min) and motor complexes (min), mean height (mN) and frequency (*n*/min). The length of the cycles is measured from the beginning of one complex to the beginning of the next complex. Electromyogram is analyzed for slow waves (cycles/min), maximum amplitude of the spike bursts (mV), duration of the spike bursts (ms) and duration of the effect (min). The data before and after administration of various doses of the test compound are compared by statistical means (Student's *t*-test).

MODIFICATIONS OF THE METHOD

Itoh et al. (1977) and Nagakura et al. (1996) used extraluminal force transducers for recording contractile motility of the gastrointestinal smooth muscle in conscious dogs.

Nakada (1995) recorded gastrointestinal and gallbladder contractions in conscious dogs by chronically implanted strain gauge transducers and gallbladder volume changes by a chronically indwelling gallbladder catheter.

Orihata and Sarna (1994) investigated contractile mechanism of gastroprokinetic agents in conscious dogs. The spatial and temporal parameters of gastric, pyloric and duodenal contractions during the entire period of gastroduodenal emptying, during a 60-min period of drug infusion and during the post-drug-infusion period were analyzed by a computer method.

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J.4.4 Absorption

J.4.4.1 Everted sac technique

PURPOSE AND RATIONALE

The everted sac technique is used to study the transport of substances from the mucosal to the serosal surface (Wilson and Wiseman 1954).

PROCEDURE

Male Wistar rats weighing 150–220 g are anesthetized with 30 mg/kg pentobarbitone s.c. A midline abdominal incision is made. The rats are sacrificed by cardiac puncture. The small intestine from the ligament of Treitz to the ileocaecal junction with a length of 6–7 cm is rapidly removed and everted with a glass rod. The sac is securely ligated at both ends and filled with Krebs-Henseleit bicarbonate buffer solution (pH 7.4) containing 0.4% glucose and pre-gassed with 95% O_2/5% CO_2. The sac is incubated at 37 °C in a glass vessel containing the same buffer solution and gassed with 95% O_2/5% CO_2.

After 5 min, the drug solution is added to the glass vessel and the preparation is further incubated. After given time intervals, the fluid from the everted sac is removed and the volume determined. The concentration of the drug transported from the mucosal to the serosal side is determined by appropriate analytical methods.

EVALUATION

Determinations of the drug in the everted sac at different time intervals allow evaluation of pharmacokinetic parameters.

MODIFICATIONS OF THE METHOD

Madar (1983) used the small intestine everted sac of **chicken** for demonstration of amino acid and glucose transport.

Harnett et al. (1989) used everted segments of distal ileum of **rats** to study taurocholate absorption.

Turner et al. (1990) used everted sacs of ileum in **sheep** to study selenate and selenite absorption.

Goerg et al. (1992) used the stripped descending colon of the **rat** as everted sac to study the inhibition of neuronally mediated secretion in rat colonic mucosa by prostaglandin D_2.

Under ether anesthesia, the descending colon is removed and transferred into ice-cold bathing solution. The colon is placed on a plastic rod. After a circular incision with a blunt scalpel, the serosa and muscularis propria are stripped away, leaving the mucosal-submucosal preparation consisting of the mucosa, the muscularis mucosae, and a part of the submucosa with the completely preserved submucosal plexus. The tube-like mucosal-submucosal preparation is mounted as an everted sac in a holding apparatus (volume of the outer compartment 25 ml). The potential difference between the outer mucosal side and inner serosal side is measured by two agar bridges. The tissue is short-circuited by a voltage clamp after correcting for the offset potential and compensating for the solution resistance.

Moreover, transmural ion fluxes can be determined with the everted sac technique. Net fluxes of sodium, chloride, and potassium are studied by direct measurements of volume changes and changes of electrolyte concentrations in the inner volume of the everted sacs. Unidirectional fluxes can be determined simultaneously with the direct measurement of net fluxes by adding the isotopes $^{22}Na^+$ and $^{36}Cl^-$ to one side of the everted sac.

Schilling and Mitra (1990) used the everted gut technique in rats to study enteral insulin absorption.

Mizuma et al. (1993) studied the active absorption in the intestine and metabolism of the β- and α-anomers of the glucoside and galactoside of p-nitrophenol to find a more suitable prodrug for poorly absorbed drugs. The everted sac technique was used to investigate the intestinal absorption of these glycosides from the mucosal to the serosal side of the rat jejunum.

Kitagawa et al. (1996) investigated the influence of various factors, such as temperature, on the absorption of methochlorpromazine in the small intestinal everted sac of rats.

Tanaka et al. (1996) estimated the transport characteristics of thyrotropin-releasing hormone (TRH) and

its chemically modified derivative with lauric acid (Lau-TRH) across the rat small or large intestine by means of an *in vitro* everted sac experiment.

Sasaki et al. (1995) studied the absorption characteristics of azetirelin, a new thyrotropin-releasing hormone analogue in rats by means of *in situ* closed loop and *in vitro* everted sac experiments.

Toskulkao et al. (1995) examined the effects of stevioside (a natural non-nutritive sweetening agent) and of steviol (a product of enzymatic hydrolysis of stevioside) on intestinal glucose absorption in hamster jejunum using the everted sac technique.

Motozono et al. (1994) determined the effect of age on gastrointestinal absorption of tobramycin in suckling, weanling and adult rats by an everted sac method.

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J.4.4.2 Stomach emptying and intestinal absorption in rats

PURPOSE AND RATIONALE

Reynell and Spray (1956) described a method for the simultaneous measurements of gastric emptying, intestinal transit and absorption of test substances in the rat using phenol red as marker.

PROCEDURE

Adult male Wistar rats weighing 200–300 g are allowed water but are deprived of food 24 h before the experiment. They are treated orally or subcutaneously with the test compound 15 min prior to oral administration by gavage of 1.5 ml 0.07% phenol red in 2% carboymethylcellulose solution. Fifteen min later the animal is sacrificed and the stomach is immediately removed. The whole stomach including the stomach content is alkalized with 1 N NaOH and homogenized. The homogenate is filtered, and after precipitation of the protein with 10% trichloroacetic acid, centrifuged for 15 min at 3 000 rpm. The concentration of phenol red in the supernatant is measured colorimetrically in a photometer at 546 nm.

EVALUATION

Percentage of stomach emptying (S_e) is calculated according to the following formula:

$$S_e = 100 - P_s \times P_a^{-1} \times 100$$

- P_s = Concentration of phenol red in the stomach (µg/ml)
- P_a = Concentration of phenol red in the initial solution after addition of equal volumes of 1 N NaOH and trichloroacetic acid (µg/ml)

MODIFICATIONS OF THE METHOD

By selective ligations of the different parts of the gastrointestinal tract and analysis for phenol red contents and for drug substance, as well as by sacrificing the animals after various time intervals, the degree and time course of absorption can be studied.

Droppleman et al. (1980) described a simplified method for assessing drug effects on gastric emptying in **rats**. Three ml of a semi-solid test meal, based on

methylcellulose, are given to rats fasted 24 h prior to the experiment. At a specified time following the test meal, the rats are sacrificed, laparatomized, and the stomachs removed. The full stomachs are weighed on an analytical balance; they are opened and rinsed. Excess moisture is removed and the empty stomach weighed again. The difference is subtracted from the weight of 3 ml of the test meal, indicating the quantity emptied from the stomach during the test period. Gastric motor stimulants, e.g., metoclopramide increase, and anticholinergic compounds decrease gastric emptying.

Megens et al. (1990) used phenol red as marker to measure gastrointestinal propulsion after castor oil or paraffin oil challenge in **rats**.

Hedge et al. (1995) studied 5-HT_4 receptor mediated stimulation of gastric emptying in rats using a specially prepared semi-solid test meal containing charcoal.

Bonnafous et al. (1995) investigated benzodiazepine-withdrawal-induced gastric emptying disturbances in rats. Male Wistar rats, weighing 200–250 g, fasted for 16 h, received by gavage 2 ml of a test meal containing 1 µCi/ml of ^{51}Cr sodium chromate, 15 min after drug administration. Thirty min later, the animals were sacrificed by cervical dislocation. The stomach, small intestine (10 segments) and the colon were excised and placed into tubes. Radioactivity was determined by placing the tubes in a gamma counter. Gastric emptying was calculated as the percentage of total counts found in the small intestine and the colon.

Varga et al. (1995) determined gastric emptying in rats 5 min after a 3-ml intragastric load of 0.9% NaCl using phenol red as marker in order to define which bombesin receptors are involved in the delay of gastric emptying by bombesin-like peptides.

Lasheras et al. (1996) studied gastric emptying in rats. Sixty min after oral administration of vehicle or test compounds, the rats received by gavage 40 steel spheroids (1 mm diameter) in 2 ml 3% carboxymethylcellulose. Sixty min later, the animals were sacrificed and the spheroids remaining inside the stomach counted.

Yegen et al. (1996) studied the inhibitory effects of gastrin releasing peptide on gastric emptying in rats using methyl cellulose and phenol red as non-absorbable marker.

Haga et al. (1994) studied gastric emptying in **mice**. Male mice, weighing 18–22 g, had free access to food and water before the experiment. The test compounds were administered orally in 10 ml/kg 0.5% methylcellulose solution. The mice were deprived of food an water and sacrificed 4 h later by cervical dislocation. The stomachs were removed and opened. The contents of the stomach were mixed with 10% trichloroacetic acid, and centrifuged at 3 000 rpm for 30 min. The weight of the sediment was taken as the food remaining in the stomach.

Ding and Håkanson (1996) examined the effect of drugs on a cholecystokinin-A receptor-mediated response by gastric emptying of a charcoal meal in mice.

Costall et al. (1987) used the **guinea pig** to study the influence of a 5-HT_3 antagonist on gastric emptying.

Brighton et al. (1987) used scintigraphy following indium-111-labeled meals in Beagle **dogs** and **baboons**. Indium-111-labeled polystyrene beads (500 mCi per dog) were mixed into a meal consisting of 50 g of finely crushed commercial dog food and 50 ml of milk. Images of 1 min duration were taken every 5 min for a period of 1 h using a large field of view gamma camera (ON Sigma 410).

Gullikson et al. (1991, 1993) studied gastric emptying of a solid meal in dogs.

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J.4.4.3 Intestinal drug absorption

PURPOSE AND RATIONALE

Doluisio et al. (1969) described an *in situ* rat gut technique to determine absorption rates of drugs.

PROCEDURE

Male Sprague-Dawley albino rats weighing 220–260 g are fasted 16–24 h prior to surgery allowing, however, drinking water ad libitum. They are kept in cages having wide mesh floors to prevent coprophagia. One hour prior to surgery, the animals are anesthetized with 1 g/kg ethylurethane i.p. The small intestine is exposed by a midline abdominal incision, and two L-shaped glass cannulae are inserted through small slits at the duodenal and ileal ends. Care has to be taken to handle the small intestine gently in order to maintain an intact blood flow. The cannulae are secured by ligation with silk suture, and the intestine is returned to the abdominal cavity. Four cm long segments of tubings are attached to the exposed ends of both cannulae, and a 30-ml hypodermic syringe fitted with a three-way stop-cock and containing perfusion fluid warmed to 37 °C is attached to the duodenal cannula. As a means of clearing the gut, perfusion fluid is passed slowly through it and out the ileal cannula to be discarded until the effluent is clear. The remaining perfusion solution is carefully expelled from the intestine by means of air pumped through from the syringe.

Immediately afterwards, 10 ml of drug solution are introduced into the intestine by means of a syringe. The stopwatch is started, and the ileal cannula is connected to another 30 ml syringe fitted with a three-way stopcock. This arrangement enables the operator to pump the lumen solution into either the ileal or the duodenal syringe, remove 0.1-ml aliquots, and return the remaining solution to the intestine within 10–15 s. To assure uniform drug solution concentrations throughout the gut segment, aliquots are removed from the syringes alternatively. Samples are collected every 5 min. Depending on the drug to be studied, the concentration is determined by chemical methods.

EVALUATION

The concentrations of the drug are plotted on a logarithmic scale on the ordinate versus time on the abscissa on a linear scale. Half-life values can be calculated.

MODIFICATIONS OF THE METHOD

Ochsenfahrt (1979) described a more sophisticated method to measure the absorption of drugs in the vascularly perfused, isolated intestine of the rat. Male rats weighing about 350 g are anesthetized with urethane. The abdomen is opened by a midline incision. The mesentery of the ascending and transverse colon is gently pulled away from the mesentery of the small intestine. The vessels to the colon are ligated and cut. The superior mesenteric artery is freed from the surrounding mesentery. The duodenal vessels are ligated and cut. A suitable segment of the jejunum of about 7 cm length is selected. The proximal cannula is tied into the lumen of this segment, and the duodenum is cut. The lumen of the segment is washed with 3–4 ml warm Krebs-Henseleit solution. Then, the distal cannula is tied. After the mesenteric vessels distal to the experimental segment are ligated, the rest of the small intestine, ileum, and colon is excised. Temporarily, the intestine is covered with gauze soaked with saline solution.

The preparation of the rat is interrupted for 8–10 min, while a second rat (donor rat), which has been anesthetized with urethane, is prepared. The donor rat supplies the arterial blood; the spontaneous respiration is supported with a respiration pump through a tracheal cannula. The right jugular vein of the donor rat is cannulated for the blood infusion and 2 mg heparin dissolved in 0.1 saline solution is administered. A cannula is placed into the left carotid artery of the donor rat; this tube is later connected to the superior mesenteric artery of the test segment. Blood lost by the donor rat is replaced with heparinized blood taken from other rats immediately before the beginning of the experiment.

In the test animal, a thin silicon tube is inserted in the left carotid artery. Two mg heparin, dissolved in 0.1 ml saline, are injected. The superior mesenteric vein is ligated distal to the splenic vein and a plastic cannula is inserted for venous outflow. The cannula is then connected to a drop counter. The luminal cannulae of the segment are connected to a recirculation unit which is filled with Krebs-Henseleit solution at 37 °C, and the luminal perfusion of the segment (mucosal solution) is started. The mucosal solution is oxygenated and recirculated. The superior mesenteric artery is ligated with a thread at its origin at the aorta and then cannulated. This cannula is first connected to the carotid artery of the test animal and later to the carotid

artery of the donor animal. The isolated vascularly perfused segment is suspended in a serosal bath containing Krebs-Henseleit solution at 37 °C. Venous blood outflow is collected in plastic vials at 15 min intervals. Samples are taken from the mucosal and serosal solutions at the beginning and the end of each period. By this way, the mucosal disappearance rate, the venous appearance rate, and the serosal appearance rate can be measured.

Schilling and Mitra (1992) measured insulin absorption from the distal duodenum/proximal jejunum and from the distal jejunum/proximal ileum in anesthetized rats by the closed-loop technique.

Schümann and Hunder (1996) described a modified device for the differentiated study of intestinal transfer in isolated intestinal segments from mice and suckling rats *in vitro*. A luminal perfusion system for small intestinal segments was adapted for the use in mice and rat pups to investigate longitudinal differences in drug and toxin transfer.

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J.4.5 Duodenal ulcer formation

J.4.5.1 Cysteamine-induced duodenal ulcers in rats

PURPOSE AND RATIONALE

Duodenal ulcers can be induced in rats by repeated administration of cysteamine or propionitrile in rats (Dzan et al. 1975; Szabo 1978; Szabo et al. 1979).

PROCEDURE

Male Sprague-Dawley rats with an initial weight of 200 g are used. Cysteamine-HCl is administered three times on day 1 in a dose of 280 mg/kg orally. Protective drugs, such as H_2-antagonists, are given 30 min prior cysteamine treatment. The rats are sacrificed on the third day. For histological evaluation, the stomach and duodenum are fixed in 10% aqueous buffered formaldehyde and paraffin-embedded sections are stained with hematoxylin and eosin. Duodenal ulcers develop in the anterior (antimesenteric) and posterior wall of the proximal duodenum, about 2–4 mm from the pylorus. The more severe ulcers, located on the anterior wall, frequently perforate, resulting in focal or generalized peritonitis, or penetrate into the liver. The opposite ulcer invariably penetrates into the pancreas.

EVALUATION

The intensity of the duodenal ulcer is evaluated using scores from 0 to 3.

- 0 = no ulcer
- 1 = superficial mucosal erosion
- 2 = deep ulcer usually with transmural necrosis
- 3 = perforated or penetrated ulcer

CRITICAL ASSESSMENT OF THE METHOD

In view of the development of modern gastric K^+/H^+-ATPase inhibitors the predictive value of methods using experimental ulcers in the rat for clinical healing rates in man has been challenged (Herling and Weidmann 1994).

MODIFICATIONS OF THE METHOD

The cysteamine-induced duodenal ulcer has been used for pharmacological studies by many authors, e.g.: Evangelista et al. (1992), Krantis et al. (1993), Pascaud et al. (1993), Tanaka et al. (1993), Morimoto et al. (1994), Pendley et al. (1995), Sikiric et al. (1997), Drago et al. (1999).

Okabe et al. (1971), Sato et al. (1989) described a method for experimental, penetrating gastric and duodenal ulcers in rats. Rats were anesthetized with ether and an incision was made in the abdomen. A round metal mold, 6 mm in diameter, was placed in close contact with the serosal surface of the duodenal wall, about 7 mm distal to the pylorus. **Glacial acetic acid** (60 ml) war poured into the mold and was left in place for 20 s. After the acetic acid was removed, the treated surface was rinsed with 50 ml of 0.02 N NaOH and the abdomen was closed. A drug or the vehicle was given p.o. once a day for 14 consecutive days beginning 2 days after the operation. The animals were sacrificed on the 16th day after the operation and the ulcerated area (mm^2) was measured.

Mepirizole-induced duodenal ulcers were described by Okabe et al. (1982), Sato et al. (1989), Tanaka et al. (1989). A drug or the vehicle was given p.o. 30 min before mepirizole (200 mg/kg) was administered s.c. Twenty-four hours later, 1 ml of 0.5% Evan's blue solution was injected via the tail vein of each rat and the rats were sacrificed by CO_2 asphyxiation. The gastroduodenal region was removed and examined for lesions.

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J.4.6
Models of inflammatory gut disease

J.4.6.1
Experimental ileitis

PURPOSE AND RATIONALE

An experimental model of inflammatory bowel disease produced by the intraluminal administration of the cytotoxic plant lectin ricin into the rabbit ileum was developed by Sjogren et al. (1994), Goldhill et al. (1995, 1997). *In vitro*, electric field stimulation results in larger non-cholinergic excitatory junction potentials in ricin-treated circular muscles than in controls.

PROCEDURE

Inflammation

Acute ileitis is induced with ricin in male New Zealand white rabbits. The animals are anesthetized with intramuscular xylazine (9 mg/kg) and ketamine (50 mg/kg) and maintained with intravenous pentobarbital (15 mg/kg). A midline incision is made, a ligated terminal ileal loop (~10 cm in length) is constructed in each animal and 1 ml of ricin (1 mg/ml) or vehicle is injected into the lumen. The loop is removed after 5 h, a time period that allows development of ileitis, abnormal myoelectric activity and increased response to electric field stimulation. Animals are sacrificed with an overdose of pentobarbital. The loop is opened along its length, gently flushed of luminal contents with cold oxygenated Krebs-bicarbonate-saline and prepared for contractility studies.

Contractility studies

Muscle strips (~1×0.4 cm) with mucosa removed are cut in the axis of the circular muscle and attached to isometric tension transducers in 10-ml organ baths with modified oxygenated Krebs-bicarbonate-saline at 37.5 ±0.5°C. Tissues are allowed to equilibrate at L_i (the length at which no tension can be measured) for 20 min. The strips are then stretched to L_0, which is

determined as the length at which maximum force is generated in response to acetylcholine (0.5–1 mM). Strips are then allowed to equilibrate for an additional 20 min.

Effect of inflammation on response to tachykinins

Concentration-response curves are constructed to substance P and neurokinin agonists (or analogues) on separate muscle strips in vehicle- and ricin-treated tissues. Studies are performed in the presence or absence of tetrodotoxin to distinguish between neural and non-neural effects of ricin treatment.

Effects of ricin treatment to responses to electrical field stimulation

Muscle strips are passed through a pair of ring electrodes (2-mm diameter) and stimulated for 10 s by square-wave pulses (0.5 ms duration, supramaximal voltage) at 1–10 Hz. Stimulation is performed in the presence of atropine (1 mM) and N^G-nitro-L-arginine methyl ester) (L-NAME) (0.1 mM).

EVALUATION

Maximum increases in muscle tone in response to tachykinin addition of electrical field stimulation are obtained through visual analysis of chart recorder outputs. Responses to tachykinins are expressed as absolute tension development. Electrical field stimulation data are expressed as a percentage of the response to 1 μM acetylcholine added at L_0 to reduce the variation of the data. Values are given as mean ±SEM Tachykinin concentration responses are fitted to sigmoid curves and EC_{50} values (with 95% confidence intervals) are determined from these curves. Differences between frequency or concentration-response curves are assessed statistically by multivariate analysis of variance, with adjustments made for multiple comparisons. In cases in which curves are significantly different to one another, maximal responses were compared statistically using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Miller et al. (1991) induced ileitis in **rabbits** by luminal perfusion with histamine monochloramine or with acetic acid.

Rachmilewitz et al. (1997) induced inflammation in the small intestine in **rats** by intrajejunal administration of 0.1 ml 2% iodoacetamide.

Sukumar et al. (1997) induced ileitis in rats by two doses of indomethacin (7.5 mg/kg) administered subcutaneously 24 h apart.

Ileitis in **guinea pigs** was induced by intraluminal trinitrobenzene sulfonic acid (Miller et al. 1993; Izzo et al. 1998; Mazelin et al. 1998).

Likewise, ileitis in **hamsters** was induced by intraluminal injection of trinitrobenzene sulfonic acid (Boyd et al. 1995).

Shibata et al. (1993) induced ileitis in **dogs** by administration of 10 ml 100% ethanol and 1 g trinitrobenzene sulfonic acid dissolved in 10 ml water through a tube inserted into the ileum.

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J.4.6.2 Experimental colitis

PURPOSE AND RATIONALE

Inflammatory bowel diseases, ulcerative colitis and Crohn's disease, represent chronic alteration of the gastrointestinal tract of unknown etiology perhaps involving immunological events. The immunological parameters have been described as secondary but may possibly be attributed to the chronicity of the disease. Several compounds have been described to elicit cell-mediated immune responses in the gut, such as dinitrochlorobenzene

(DNCB) (Rosenberg and Fischer 1964; Norris et al. 1982; Norris 1989) or 2,4,6,-trinitrobenzene sulfonic acid (TNBS) (Morris et al. 1989; Selve and Wöhrmann 1992).

PROCEDURE

A three-step concept is realized to mimic the human disease, using 2,4,6-trinitrobenzene sulfonic acid (TNBS) as a defined hapten:

1. specific hypersensitivity by active immunization,
2. local inflammation by local challenge,
3. chronicity by chronic application of the immunogen.

Female Sprague Dawley rats weighing 150–200 g are sensitized by intradermal injection of 0.8% TNBS (Fluka, Neu-Ulm, Germany) solution into a shaved area on the back once daily for three consecutive days. TNBS is dissolved in 0.05 ml Freund's incomplete adjuvant together with 1 mg/ml ovalbumin. After 18 days, the animals receive a further intradermal booster injection. Intradermal challenge of 0.08% TNBS in 0.05 ml 0.9% NaCl solution with or without ovalbumin, or ovalbumin solution without TNBS, is given 14 days later in order to determine the type and specificity of the immunological reaction.

Ten days after the intradermal challenge, a flexible polyethylene tube of 0.5 mm diameter is implanted under ketamine (100 mg/kg i.p.) anesthesia 15 cm proximal to the cecum and emerging at the neck for TNBS or drug administration. After a 10-day recovery phase, the animals are treated daily for 3 weeks with 0.08% TNBS in saline (0.2 mg/rat) given through the catheter. Control groups receive only saline. Drugs are applied either by gavage twice a day. suspended in carboxymethyl cellulose, or intraluminally once a day, suspended in saline. The animals are sacrificed by CO_2 inhalation 24 h after the last intraluminal application of TNBS. The distal 10 cm of small intestine anterior to the ileo-caeco-colic junction (5 cm distance to the open end of the catheter) including Peyer's patches are dissected, cut open longitudinally and rinsed with saline.

Immediately after dissection, the distal small intestine is visually assessed for inflammation according to the following scores:

Enteritis score	*Gross morphology*
0	No visible damage of the whole 10 cm the small intestine
1	Slight inflammation, slight redness (hyperemia), villi visible under 15 fold magnification
2	Intermediate inflammation, discontinuous hyperemia, intermediate redness of villi
3	Intensive inflammation, intensive hyperemia, intensive redness of villi

The dissected gut segments, precisely 10 cm long, are weighed for measurement of edema formation. They are then incubated in Tris buffer for 30 min at 37 °C in a shaking water bath (1 ml/100 mg tissue, Tris 50 mM, pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 1 mg/ml glucose). Following incubation, aliquots of the solutions are centrifuged (13 000 *g*, 2 min, 20 °C) for determination of leucotriene B_4 (TB_4) by a commercial radioimmuno-assay (e.g., Amersham Buchler, Brunswick).

EVALUATION

Results are expressed as means ±SEM of (*n*) experiments. Differences between control and inflamed tissue, and influence of drug treatment are compared. Statistical significance is calculated by Wilcoxon-Mann-Whitney *U*-test for unpaired data. The level of significance is taken as $p < 0.05$.

MODIFICATIONS OF THE METHOD

Several authors used the trinitrobenzene sulfonic acid model in *rats* with slight modifications (Kitano et al. 1996; Yue et al. 1996; Lora et al. 1997; Taniguchi et al. 1997; Cruz et al. 1998; Fries et al. 1998; Goldhill et al. 1998).

Zea-Ariate et al. (1994) studied chronic colitis in Wistar rats after intracolonic instillation of 20 or 42 mg of trinitrobenzene sulfonic acid in 30% and 40% ethanol and compared the effect with the administration of ethanol alone.

Alternatives to the 2,4,6-trinitrobenzene sulfonic acid model in **rats** were proposed:

Wallace et al. (1995), Hawkins et al. (1997) recommended dinitrobenzene sulfonic acid to produce experimental colitis in the rat.

Patterson and Colony (1983) tested sulphasalazine and 5-aminosalicylic acid in experimental colitis induced in **guinea pigs** by topical dinitrochlorobenzene.

Neurath et al. (1996) studied chronic intestinal inflammation induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) in **mice**.

These authors (Neurath et al. 1997) investigated the role of tumor necrosis factor in this mouse model and showed that no significant TNBS-induced colitis could be induced in mice in which the TNF-alpha gene had been inactivated by homologous recombination.

Antony et al. (1997), Oyen et al. (1997) used trinitrobenzene sulfonic acid to induce experimental colitis in **rabbits**.

Dams et al. (1998) compared gamma-camera imaging in rabbits with colitis after retrograde instillation of trinitrobenzene sulfonic acid with technetium-99m-labeled liposomes after various time intervals with macroscopically scored severity of inflammation.

A model of diffuse colitis in **rats** induced by intraluminal colonic instillation or serosal application of dilute **acetic acid** was described by MacPharson and Pfeiffer (1976, 1978). Ritzpatrick et al. (1990) tested the anti-inflammatory effects of various drugs on acetic acid induced colitis in the rat.

For instillation into the colon of rats, a 2 cm length of soft polyethylene tubing is fitted to a Luer stub adapter. The open end of the tubing is sealed with glue. The whole length of the tubing is perforated with a needle at 0.5 mm length by four holes, 90° apart. A 1 ml syringe containing 10% acetic acid in saline is fitted to the adapter. The tubing is inserted intrarectally into the colon of rats and 0.2 ml is injected into the lumen. After 10 s contact *in situ*, the remaining acid is withdrawn and the lumen washed with three successive 0.5 ml volumes of isotonic saline. Test drugs are administered daily during the following days. Diffuse colonic lesions appear after 3 days in control animals and the animals experience a bloody diarrhea. An initial mucosal inflammation develops into submucosal edema; petechial hemorrhages become enlarged with subsequent neutrophil invasion, and pseudopolyps become evident.

Terzioglu et al. (1997) studied the effect of prostaglandin E_1 on the experimental colitis induced by rectal instillation of 10% acetic acid in rats.

Fabia et al. (1994) induced colitis in rats in an exteriorized colonic segment by administration of 4% acetic acid for 15 s. Four days later, this colonic segment was examined using a morphological scoring system, and measurements of myeloperoxidase activity and plasma exudation into the colonic segment.

Eliakim et al. (1995) demonstrated that ketotifen ameliorated capsaicin-augmented acetic acid-induced colitis. Rats were pretreated with subcutaneous injections of 20, 30, and 50 mg/kg capsaicin. Colitis was induced 2 weeks later by flushing 2 ml 5% acetic acid into the proximal colon.

Higa et al. (1997) studied the role of neutrophils in the pathogenesis of acetic acid-induced colitis in mice.

Millar et al. (1996) evaluated the antioxidant potential of new treatments for inflammatory bowel disease using acetic acid-induced colitis in rats.

Myers et al. (1997) determined colonic transit in rats by calculating the geometric center of distribution of a radiolabeled marker (^{51}Cr) instilled into the proximal colon after induction of distal colitis by intracolonic administration of 4% acetic acid.

Several **other agents** can induce experimental colitis in animals (Kim and Berstadt 1992), such as

phorbol esters (Fretland et al. 1990),

carrageenan (Marcus and Watt 1969; Benitz et al. 1973; Watt and Marcus 1973; Abraham et al. 1974; Jensen et al. 1984. Kitano et al. 1994; Pricolo et al. 1996),

amylopectin-sulfate (Watt and Marcus 1972),

dextran sulfate (Ohkusa 1985; Okayasu et al. 1990; Axelsson et al. 1966; Shintani et al. 1997; Kanauchi et al. 1998),

the **chemotactic peptide FMLP** (Magnusson et al. 1985; *von Ritter et al. 1988;* LeDuc and Nast 1990).

Ekstrom (1998) described **oxazolone**-induced colitis in rats. Dark Agouti rats were skin-sensitized with oxazolone and further challenged intra-rectally with oxazolone dissolved in carmellose sodium/peanut oil.

Suzuki et al. (1997) developed a rat model for human ulcerative colitis by using **1-hydroxyanthraquinone** to cause severe inflammation of colonic mucosa.

Aiko et al. (1997), Satdnicki et al. (1998) induced chronic granulomatous colitis in female Lewis rats via intramural (subserosal) injections of **peptidoglycan-polysaccharide** into the distal colon.

Surfactants being used to enhance drug absorption may cause intestinal damage. Oberle et al. (1995) evaluated mucosal damage by surfactants in a single-pass *in situ* perfusion model in the rat. The release of LDH and mucus into the lumen of jejunum and colon following perfusion of the nonionic surfactants Tween 80 and Triton X-100 was determined.

Axelsson and Ahlstedt (1993) reviewed the actions of sulfasalazine and analogues in various animal models of experimental colitis, in particular in the hapten-, immune complex- and dextran models.

Bach et al. (1985) studied the inhibition of LTC synthetase and of rat liver glutathione *S*-transferases by sulfasalazine.

Stein et al. (1993) determined arachidonic acid oxidation and damage in the colon in rats stressed by the **cold-restraint** method.

Kirsner et al. (1959), Kraft et al. (1963) induced severe colitis in **rabbits** which had previously been sensitized to egg albumin and had mild colonic inflammation induced by intrarectal instillation of a small amount of diluted **formalin**.

Meenan et al. (1996) induced immune-complex colitis in rabbits by using various formalin concentrations (2%, 0.75%, and 0.5%).

Hodgson et al. (1978) induced colitis in rabbits by a modified technique. Preformed immune complex of human serum albumin and anti-HSA with antigen excess was injected to non-sensitized rabbits after provocation of mild inflammation in the colon with diluted formalin.

Kuroe et al. (1996a,b) induced granulomatous enterocolitis in rabbits by repeated submucosal injection

of **muramyl dipeptide** emulsion into the rectum and colon with a flexible endoscope. Extraintestinal manifestations, such as pericholangitis, were observed.

Walsh and Zeitlin (1987) studied the effects of salazopyrin, 5-aminosalicylic acid and prednisolone on immune complex-mediated colitis in **mice**.

A survey on various genetic and immune manipulations which lead to inflammatory bowel disease in mice was given by Powrie and Leach (1995).

$CD4^+$ T lymphocytes injected into severe immunodeficient (SCID) mice lead to an inflammatory and lethal bowel disease (Claesson et al. 1996; Leach et al. 1996; Rudolphi et al. 1996; Bregenholt et al. 1998).

Hermiston and Gordon (1995) described an inflammatory bowel disease in mice expressing a dominant negative N-cadherin resembling Crohn's disease.

Watanabe et al. (1998) reported that interleukin7 transgenic mice develop chronic colitis with decreased interleucin7 protein accumulation in the colonic mucosa.

Mitchell and Turk (1990) described a model of **gran-ulomatous bowel disease in guinea pigs**. Epithelioid cell granulomas and primary macrophage granulomas were induced by the inoculation of BCG (Pasteur) and irradiated Mycobacterium leprae, respectively, into the terminal ileum.

Wallace et al. (1998) induced colitis in guinea pigs and in rats by intracolonic administration of trinitrobenzene sulfonic acid.

Some **monkeys**, such as the cotton-top tamarin, *Saguinus oedipus* (Chalifoux and Bronson 1981; Madara et al. 1985; Lushbach et al. 1985; Podolsky et al. 1988; Hesterberg et al. 1996; Warren 1996), and juvenile rhesus monkeys (Adler et al. 1990), develop **spontaneous colitis**.

CRITICAL ASSESSMENT OF THE METHODS

The relevance of animal models for the pathogenesis and treatment of human inflammatory bowel disease was reviewed by Dieleman et al. (1997) and by Sartor (1997).

A critical review of *in vitro* models in inflammatory bowel disease was given by McKay et al. (1997).

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J.5
Emetic and anti-emetic activity

J.5.0.1
Assessment of emetic and anti-emetic activity in dogs

PURPOSE AND RATIONALE

Emesis comparable to man occurs only in a few animal species. Among laboratory animals, the dog is a suitable species to test anti-emetic drugs. Apomorphine-induced emesis is also used to evaluate neuroleptic drugs (see E.5.3.8). Burkman (1982) described a technique relying upon the use of apomorphine either as a reference standard against which other emetics can be compared, or as a challenging agent against which anti-emetic compounds can be evaluated.

PROCEDURE

Beagle dogs weighing between 15 and 20 kg are used. Each dog is given 200 g food 30 min prior to an assay session. The threshold emetic dose of apomorphine

hydrochloride is established for each dog by administering single doses at 5 day intervals in gradually increasing amounts. The starting dose is 0.07 mM/kg (22 mg/kg) body weight, i.m., and is subsequently increased (or decreased) as required. Injection sites alternate between contralateral gluteus muscles. After every third or fourth dose of the emetic, the animals receive an equivalent volume of vehicle under similar conditions in order to detect the presence of a conditioned emetic response.

The threshold dose is defined as the concentration provoking an emetic episode and determined for each individual animal. The threshold emetic dose is relatively stable for a given group of dogs over a period of 2 months. Continued administration to the same dogs for longer periods of time is inadvisable as Pavlovian emetic conditioning becomes evident after 8–10 doses of apomorphine. Establishment of an emetic threshold for a test compound using a similar dosing schedule allows to quantitatively express the test compound's emetic potency as a ratio compared with the reference standard. Usually, 4–6 animals are sufficient to provide a reliable estimate of the test compound's emetic efficacy and potency.

In the anti-emetic assay, dogs whose apomorphine threshold emetic dose has been determined receive various concentrations of the potential anti-emetic drug at a given time interval prior to apomorphine. The dose initially selected for the anti-emetic is a fraction of the acute LD_{50} of this drug in mice. A new threshold dose is estimated in the presence of the test anti-emetic and compared to the threshold dose in the presence of the reference standard chlorpromazine.

EVALUATION

Using the threshold doses, the relative potency of an emetic compared to apomorphine, or the relative potency of an anti-emetic compared to chlorpromazine, is calculated.

MODIFICATIONS OF THE METHOD

Cisplatin-induced emesis in the **dog**, as described by Gylys et al. (1979), was used by Turconi et al. (1991) to test the anti-emetic properties of 5-HT_3 receptor antagonists.

Gupta and Sharma (1996) tested the activity of antioxidants against emesis induced by an intravenous dose of 3 mg/kg cisplatin in healthy mongrel dogs.

Szelenyi et al. (1994) described emesis in **domestic pigs** as a new experimental tool for detection of antiemetic drugs and for evaluation of the emetogenic potential of new anticancer agents. Healthy young, 12–15-week-old domestic pigs of either sex were lightly anesthetized with ketamine (10 mg/kg, i.v.) and xylazine (2 mg/kg, i.v.) and a cannula inserted into a superficial vein of one of the extremities. The challenging agents, e.g., cisplatin, carboplatin, cyclophosphamide, or ifosfamide were infused intravenously at different doses during a total administration time of 15 min. After removing of the cannula the animals were placed in their boxes for observation of emesis over 24 h. Drugs with anti-emetic potential were given intravenously 15 min prior cisplatin infusion.

Göthert et al. (1995) found a dose-dependent inhibition of cisplatin-induced emesis in pigs by anpirtoline, a mixed 5-HT_1 receptor agonist/5-HT_3 receptor antagonist.

Gardner et al. (1995, 1998) used the **house musk shrew** (*Suncus murinus*) to test antagonism against cisplatin-induced emesis.

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J.5.0.2 Anti-emetic activity in ferrets

PURPOSE AND RATIONALE

The ferret is a well established animal model of emesis which responds to cancer chemotherapeutic agents in

a manner similar to that observed in man (Florczyk et al. 1982). The animals react with vomiting and retching after challenge with central (loperamide and apomorphine), peripheral ($CuSO_4$), or mixed central and peripheral (ipecacuanha, cisplatin) emetic stimuli. The model has been used to test the anti-emetic properties of 5-HT_3 receptor antagonists and tachykinin NK_1 receptor antagonists.

PROCEDURE

Adult male ferrets weighing 1 to 1.5 kg are randomly assigned to the different treatment groups. Each animal is anesthetized by inhalation with methoxyflurane. A jugular vein is cannulated and exteriorized from the outside of the neck. Following recovery from the anesthesia, the animals are dosed with the test drug or the standard or the vehicle 30 min prior i.v. administration of 10 mg/kg cisplatin. The numbers of retches and vomits occurring following the administration of the emetogen are recorded in each animal for 5 h. Retching is defined as rhythmic inspiratory movements against a closed glottis, and vomiting as forced expulsion of upper gastrointestinal contents.

EVALUATION

Duration of action of the compounds is assessed by determining the period of time for which the inhibitory effects remain significantly different from vehicle controls.

Statistical analysis of the data is performed by a repeated measure analysis of variance (ANOVA) followed by pairwise comparisons against control at each time period using Fisher's LSD multiple comparison test.

MODIFICATIONS OF THE METHOD

Fink-Jensen et al. (1992) reported that the excitatory amino acid receptor antagonists, 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo(f)quinoxaline (NBQX) and 6-cyano-7-nitroqinoxaline-2,3-dione (CNQX), which preferentially block non-NMDA subtypes of excitatory amino acid receptors, effectively inhibit cisplatin-induced emesis in ferrets.

Emesis in ferrets was induced by X-irradiation or oral doses of copper sulfate (Andrews and Bhandari 1993).

Watson et al. (1995) studied the anti-emetic effects of a selective NK_1 receptor antagonist using the gag reflex in ferrets. The gag reflex is mediated by mechanoreceptors in the superior laryngeal nerve, which projects to the nucleus tractus solitarius. (Mifflin 1993). The gag reflex was evoked in conscious ferrets by gentle tactile stimulation of the pharynx and larynx and was recorded as an all or none response before and after drug administration.

Furthermore, the authors induced retching in the ferret by electrical stimulation of the vagal afferents under urethane anesthesia (Andrews et al. 1990). The dorsal or ventral abdominal vagus was isolated and ligated and the central cut-end stimulated before and after drug administration.

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J.5.0.3 Assessment of emetic and anti-emetic activity in pigeons

PURPOSE AND RATIONALE

Emesis in pigeons can be induced by various agents. Formerly, the phenomenon has been used for standardization of cardiac glycosides (Hanzlik 1929). More recently, dose response curves of emesis have been determined for various agents and anti-emetic effects were evaluated (Wolff and Leander 1994, 1995).

PROCEDURE

Male White Carneaux pigeons are kept in individual stainless steel cages at constant temperature and humidity. They are maintained at 90% of their free-feeding body weights by once-daily feeding of approximately 20 g Purina Pigeon Checkers.

All testing is conducted during the illuminates phase of the light-dark cycle. On test days, the birds are fed 5 min before the start of an emetic trial. If vomiting occurs, the pigeons are given an additional 20 g of feed before being returned to their home cages at the conclusion of the observation period. Individual subjects are allowed a recovery period of at least 3 days between each drug test.

For the following compounds emetic doses are reported:

- Cisplatin 10 mg/kg, injected into a wing vein.,
- Ipecac syrup, 1 to 3 ml/kg, administered via a feeding needle passed through the crop to the opening of the proventriculus,
- emetine, 1 to 20 mg/kg, injected into the pectoralis muscle,
- m-(chlorphenyl)-biguanide (mCBG), 0.32 to 5 mg/kg, injected intramuscularly,
- ditolyganidine (DTG), 5.6 mg/kg, injected intramuscularly.

Test substances with potential anti-emetic activity are injected at various doses 15 min before the emetic challenge. The animals are observed for vomiting during 2 h.

EVALUATION

ED_{50} values for with 95% confidence limits are calculated for the activity of emetic substances, as well as for the inhibition of emesis by anti-emetic drugs after a high dose of the emetic compound.

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J.5.0.4 Activity against motion-induced emesis

PURPOSE AND RATIONALE

The house musk shrew (*Suncus murinus*) is a small insectivore that has been shown to exhibits emesis when exposed to linear reciprocation motion (Ueno et al. 1988; Okada et al. 1994).

PROCEDURE

Adult male (body weight range 55–90 g) and female (body weight range 35–50 g) are used. The animals receive a dose of the test drug or vehicle in a volume of 4 ml/kg 15 min before motion testing. The animals are placed in a Perspex chamber (11 cm wide × 22 cm long

× 11 cm high) that is attached to the platform of a shaker set to execute a linear horizontal movement of 4 cm at a frequency of 1 Hz along the long axis of the chamber. The animals are allowed approximately 3 min to become accustomed to the chamber before exposure to motion for a period of 5 min, during which the number and timing of emetic episodes are recorded. An emetic episode usually consists of a short period of rapid retching followed by a vomit. A cross-over design is used for the experiment, with animals exposed to motion testing following treatment with vehicle control on one occasion, and following treatment with test drug on another. An interval of 12 days is allowed between treatments.

EVALUATION

Group results are expressed as mean ±SEM. Either Student's *t*-test or the Wilcoxon signed rank test is used as a measure of significance.

MODIFICATIONS OF THE METHOD

Gardner and Perren (1998) described a model of post-anesthesia-induced emesis in Suncus murinus.

Lucot (1989) used cats to test the activity of HT_3 antagonists against motion-induced sickness.

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J.5.0.5 Foot tapping in gerbils

PURPOSE AND RATIONALE

Foot tapping in gerbils, a centrally mediated behavior (Graham et al. 1993; Bristow and Young 1994; Rupniak and Williams 1994; Vassout et al. (1994), has been claimed to be highly predictive for NK_1 antagonists to prevent cisplatin-induced retching in ferrets and to be a simple *in vivo* assay for CNS penetration (Rupniak et al. 1997).

PROCEDURE

Mongolian gerbils of either sex weighing 35–70 g are anesthetized by inhalation of an isoflurane/oxygen mixture to permit exposure of the jugular vein through a skin incision in the neck, using blunt dissection to clear surrounding salivary gland and connective tissues. Test compounds or vehicle are administered using an injection volume of 5 ml/kg i.v. The wound is closed and a second incision is made in the midline of the scalp to expose the skull. The highly selective, peptidase-resistant NK_1 receptor agonist GR73 632 (D-Ala-[D-Pro^9,Me-Leu^8]substance P-(1-17) (Hagan et al. 1991) is infused directly into the cerebral ventricles (3 pmol in 5 µl i.c.v.) by vertical insertion of a cuffed 27-gauge needle to a depth of 4.5 mm below bregma. The scalp incision is closed and the animal allowed to recover from anesthesia in a clear Perspex observation box (25 × 20 × 20 cm). The duration of hind foot stepping is then recorded continuously for 5 min using a stopclock. The time relapse from induction to recovery from anesthesia, with intervening i.v. and i.c.v. injections is about 3–4 min.

EVALUATION

Data are subjected to one-way analysis of variance (ANOVA), followed by Dunnett's or Newman-Keuls multiple comparison *t*-tests.

CRITICAL ASSESSMENT OF THE METHOD

The specificity of the method to predict anti-emetic activity has to be proven.

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J.6 Gall bladder functions

J.6.1 Bile secretion

J.6.1.1 Cholagogic activity in mice

PURPOSE AND RATIONALE

A rapid method for standardization of cholagogues in mice by simple weighing the gall bladder filled with bile was published by Litvinchuk (1976).

PROCEDURE

Groups of 10 female mice (e.g., NMRI strain) weighing 15–20 g are used. Food, but not water, is withdrawn 24 h prior to the experiment. The test compound or the control solution is administered subcutaneously or orally. After 1 h, the animals are sacrificed and bled from the carotid artery. Laparatomy is performed, the liver exposed, and a No. 75 silk ligature is tied around the cystic duct, which is detached from the bile ducts and removed from the peritoneal cavity. If a large volume of bile has been accumulated, the full gall bladder is removed together with the bile ducts. The isolated gall bladder is weighed on a suitable balance, after which the contents are removed, the gall bladder walls are washed with distilled water, dried on filter paper, and the organ is weighed again. The difference in weight of the full and the empty gall bladder indicates the quantity of bile secreted during a measured time. The concentration of cholates, bilirubin, and cholesterol in the bile can be determined.

EVALUATION

The average of secreted bile in groups of 10 treated mice is compared with the average value of the control group using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The method has the clear advantage of simplicity but does not measure the true bile excretion since the outflow from the bile bladder during the test period is neglected.

MODIFICATIONS OF THE METHOD

Sterczer et al. (1996) studied the effect of cholagogues on the volume of the gallbladder in healthy dogs fasted for 24 h by two-dimensional ultrasonography. The volume was measured immediately before the administration of each test substance and at 10-min intervals for 120 min thereafter.

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J.6.1.2 Choleretic activity in rats

PURPOSE AND RATIONALE

In contrast to other animals, rats do not possess a bile bladder. Therefore, cannulation of the bile duct in rats can be used as a suitable model to measure choleresis, i.e. bile production.

PROCEDURE

Male rats (e.g. Sprague-Dawley strain) weighing 300–500 g are used. Food, but not water, is withdrawn 18 h prior to the experiment. The animals are anesthetized with 5 ml/kg 25% urethane solution. The trachea is cannulated and the abdomen opened by a midline incision. The pylorus is ligated and the bile duct cannulated from the duodenum with a thin (0.05 mm diameter) polyethylene catheter which is pushed up to the liver. The secreted bile volume is measured for 30 min intervals and then the bile is returned to the duodenum. After a preperiod of 60 min, the test substances are administered subcutaneously or intraduodenally. The bile volume is registered in 30 min intervals for 2 h.

EVALUATION

The average values of the post-drug periods are compared with the pre-drug readings.

MODIFICATIONS OF THE METHOD

Several authors tested the choleretic activity of plant extracts and essential oils (De la Puerta et al. 1993; Peana et al. 1994; Trabace et al. 1994) and of synthetic compounds (Grella et al. 1992; Paglietti et al. 1994) in rats.

Tripodi et al. (1993) investigated the anticholelithogenic and choleretic activities of taurohyodeoxycholic acid by measurement of biliary flow and biliary solids content in rats.

Bouchard et al. (1993) induced cholestasis in rats by treatment with 17-α-ethinyl estradiol and studied

the influence of oral treatment with ursodeoxycholic and tauroursodeoxycholic acids.

Vahlensieck et al. (1995) studied the effect of *Chelidonium majus* herb extract on choleresis in the isolated perfused rat liver.

Miki et al. (1993) investigated the metabolism and the choleretic activity of homochenodeoxycholic acid in **hamsters** with bile fistula.

Pesson et al. (1959) recommended the **guinea pig** as the best choice among the common laboratory animals to study choleretic agents.

Cohen et al. (1992) reported a study in male **black-tailed prairie dogs** (*Cynomys ludovicianus*) weighing 1.0 ±0.2 kg anesthetized with 20 mg/kg xylazine i.m. and 20 min later with 100 mg/kg ketamine i.m. Through an abdominal incision, the cystic duct is ligated, and gallbladder bile is aspirated. A PE-50 polyethylene cannula is inserted into the common bile duct and secured with silk sutures, thereby completely diverting bile flow for collection. The bile duct cannula is externalized, the abdominal incision closed, and the prairie dog placed in a restraining cage with access to food and water.

Matsumura et al. (1996) analyzed hypercholeresis in **dogs** with pigment gallstones after cholate infusion.

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J.6.1.3 Chronic bile fistula in rats

PURPOSE AND RATIONALE

Most of the techniques for collection of bile in rats use restrained or anesthetized animals. Such factors as well as the surgical intervention itself may profoundly influence the results. Therefore, Remie et al. (1990, 1991) developed a technique for a permanent double bile fistula in rats. The procedure is described in detail.

PROCEDURE

Preparation of cannulae

Cannulae are made of silicon rubber. The proximal bile cannula, which will be inserted into the common bile duct in the direction to the liver, is 18 cm long (Silastic tubing, Dow Corning, no. 605-135; 0.51 i.d. and 0.94 o.d.) and has one square cut and one bevelled end. Two silicon rings are wrapped around the cannula at 7 mm and 50 mm, respectively, from the bevelled end.

The distal bile cannula, which will be inserted into the common bile duct in the direction of the gut, is made of the same material, is also 18 cm long and has one square cut and one bevelled end. This cannula, however must have a smaller tip-diameter (Silastic tubing, Dow Corning, no. 605-105: 0.31 i.d. and 0.64 o.d.). To serve this purpose, the square cut end of the cannula is immersed in ether, causing the tubing to dilate. When the tubing is wide enough, a 13 mm piece of small diameter Silastic tubing is inserted. Subsequently, two silicon rings are wrapped around the cannula, one at the joint of the two tubes and the other 5 cm from the tip. The tip in then cut at a 45° angle, 7 mm from the first silicon ring.

The duodenal cannula (Silastic tubing, Dow Corning, no. 605–135) is also 18 cm long, and has one square and one bevelled end. An additional ring is placed 30 mm from the tip. Before the cannulae are fixed to the skull, they must be connected to a stainless steel needle bent in a 90° angle.

Anesthesia
The animal is anesthetized with halothane/N_2O/O_2.

Preparation of the crown of the head
The head of the animal is shaved and disinfected. An incision of about 1 cm is made and the bregma exposed. Three stainless steel screws (1.0 × 4.2 mm) are mounted in the crown, two in the left and one on the right side of the bregma. The screws are tightened that approximately 2 mm is left between the skull and the head of the screws.

Double cannulation of the bile duct
The abdominal wall is shaved and disinfected and the animal secured on the operation board with adhesive tape. A midline incision is made from the level of the pubic bones to the xyphoid cartilage. The abdomen is then opened by making an incision over the linea alba towards the sternum up to the distal part of the fourth sternebra, thus exposing the xiphoid cartilage.

Then, the intestines are lifted out and are laid next to the animal on moistened gauze. Using jewelers forceps, the bile duct is stripped off its surrounding tissue and ligated with a 7-0 suture. The duct is placed under tension with an artery forceps for cannulation. With the aid of a microscope, a V-shaped hole is made just cranial of the first ligature with iridectomy scissors. The sterile proximal cannula is inserted into the duct. The second ligature is tied and pulled tight ensuring that the cannula is not obstructed. The bile is now flowing into the cannula. The first ligature is released and the threads are tied behind the silicon ring. The rat is then turned and the ligature reclamped, thereby putting the distal part of the duct under tension. A third ligature is loosely introduced around the duct, distal to the first ligature. Another V-shaped aperture is made between the first and third ligature for insertion of the distal bile cannula. The third ligature is tied and pulled tight. The first ligature is released from the artery forceps and tied around the second cannula behind the silicon ring. All the loose threats are cut close to the knots. The sections of the cannulae which lie between the silicon rings are placed kink-free in the abdominal cavity. The cannulae are fixed using 7-0 silk suture to the abdominal muscle near the xiphoid cartilage.

Cannulation of the duodenum
After location of the place where the bile duct enters the duodenum (sphincter of Oddi), a four fine-stitch purse-string suture (7-0) is made in the wall of the duodenum at the outer border at about 1 cm proximal to the sphincter. Using a 20G needle, an incision is made inside the purse string. The cannula is inserted into the duodenum until the first, smaller silicon ring has entered the lumen, and the purse string is tightened between the first and the second ring. This cannula together with the bile cannula is placed kink-free in the abdominal cavity and anchored to the internal muscle.

The abdomen is closed of resorbable sutures leaving 1 cm of the skin unclosed.

Subcutaneous tunneling and anchoring of the cannulae
From the back of the neck, a slender needle holder is pushed subcutaneously through the connective tissue in caudal direction as near as possible to the skin down to the xiphoid cartilage. The cannulae are then grasped and pulled through to emerge at the crown of the head. The abdominal wall is closed completely.

With a 5 cm piece of polyethylene tubing (0.75 × 1.45 mm), the two long ends of the L-shaped stainless steel adapters are connected and the short ends inserted into the respective cannulae. The cannulae together with the tubing are fixed to the skull with acrylic glue flowing under the heads of the screws.

Postoperative care
The animals are allowed to recover in a warm and quiet place. They reach usually preoperative weight within 2–3 days, and display normal feeding and drinking behavior. Supplementation with saline besides the normal tap water may be necessary.

Collection of bile
The animals are housed in individual metabolic cages. For bile collection, they are attached to long swiveled PE-cannulae (0.75 × 1.45 mm). A stainless steel coil is used to protect the rats from gnawing on the tubing. For continuous collection of bile, the cannula can be connected to a fraction collector.

CRITICAL ASSESSMENT OF THE METHOD
Among other applications, the method is suited to study the enterohepatic circulation of compounds.

MODIFICATIONS OF THE METHOD
Castilho et al. (1990) studied the intestinal mucosal cholesterol synthesis in rats using a chronic bile duct-ureter fistula model. Male Wistar rats weighing 300–350 g were anesthetized with 50 mg/kg pentobarbital i.p. and submitted to a bile duct-right ureter fistula utilizing a PE-50 catheter after a right-kidney nephrectomy.

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J.6.1.4 Chronic bile fistula in dogs

PURPOSE AND RATIONALE

Herrera et al. (1968) described a special cannula which can be used to obtain bile or pancreatic juice from a duodenal pouch after appropriate surgical procedures.

PROCEDURE

Male Beagle dogs weighing 15–20 kg are used. The abdomen is opened through a midline epigastric incision under barbiturate anesthesia. The duodenum is mobilized at the pyloric and jejunal ends, and a 5-cm duodenal segment containing the common bile duct is isolated. The distal stoma of the duodenum is closed and continuity restored by end-to-side duodeno-jejunostomy. The duodenal pouch is closed at both ends.

The cannula to be inserted is made of stainless steel and consists of 3 parts. The main casement measures 10.5 cm in length, with an external diameter of 1.0 cm. The internal end is flanged, and 1.5 cm from this point there is a short lateral limb, also 1.5 cm in length. The lateral limb is also flanged but, in addition, possesses a small V-shaped defect to facilitate insertion into the pouch. When not in use, the cannula is sealed from the exterior by inserting a threaded plug which allows bile to enter the duodenum in the normal manner. For collection of bile this plug is removed and a long obturator is inserted. The latter effectively isolates the bile secretion from duodenal contents. A similar hollow obturator is reserved for use when duodenal perfusion is studied, the obturator being connected via a plastic tube to the irrigating fluid.

Through a small antimesenteric incision in the duodenal pouch, the lateral limb of the cannula is inserted; the V-shaped defect in the flange facilitates entry into the pouch. A purse string secures the cannula in position. The defunctioned loop of duodenum is then brought anterior to the pancreas, and the remaining limb of the cannula inserted through a small duodenotomy and secured by a further purse-string suture. The whole system is then generously wrapped in omentum and the cannula exteriorized through a stab incision toward the flank. An external collar stabilizes the cannula. The cannula is left open to drain blood and secretions for 24 h postoperatively, after which time the plug is inserted. Physiologic saline is administered subcutaneously for a period of 3 days, after which the animals are permitted to drink water. Daily checks of the cannula are advisable to ensure that the plug remains tight. The animals receive normal kennel food and water ad libitum.

The dogs are allowed at least 4 weeks to recover. Eighteen hours prior to the experiment food is withdrawn but water allowed ad libitum. The long hollow obturator is inserted and bile collected for 15 min periods. After 1 h pretest time, the test compound is given either orally or intravenously.

EVALUATION

Secretion of bile is measured at 15 min intervals and volume and bile contents are determined from 1 ml samples. The values are compared with pretest data. The remaining bile is re-infused into the duodenum via the hollow obturator.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described several techniques for fistulae of the gall bladder and also for the fistula of the ductus choledochus in dogs.

An abdominal incision about 10 cm is made on the median line. The duodenum is pulled out and the orifice of the large (first) pancreatic duct is found. The orifice of the ductus choledochus with the orifice of the small (second) pancreatic duct is situated on the other side of the intestine some 2 or 3 cm nearer the stomach. The ductus choledochus goes straight from the gallbladder to the duodenum; further it lies parallel to is and at its end it is attached to the wall of the duodenum. The small pancreatic duct goes from the gland straight to the duodenum.

At the very beginning of the operation it is useful to cut the ligamentum that goes from the liver to the duodenum, because this facilitates orientation and operating. It is necessary to cut out a piece of the intestinal wall with the orifice of the ductus choledochus. But before this one must prepare off a little bit the intestine from the pancreas so as to be able to close conveniently and securely the hole in the intestine and divide between double ligatures the second pancreatic duct.

On the duodenum around the orifice of the ductus choledochus an incomplete oval figure is now marked with a knife, so that the duct enters this figure through the incomplete part of the oval and has its orifice in the middle of this figure. The length of the oval is about 1.5 cm and its width 1 cm. A suture is then made on the edge of this oval, which is cut out not completely but leaving a small bridge about 0.5 cm wide between the intestine and the oval; through the bridge the duct enters the oval. The mucosa of this bridge must be completely destroyed with a knife.

The oval piece of the intestine is now turned with the mucosa up and its serosa is sutured to the serosa of the intestine The hole in the intestine is very carefully closed with two layers of sutures. Two heavy threads are then passed underneath the intestine on either side of the place of operation; they are laid through the abdominal wall and tied after the operation is over. They serve as temporary supporting sutures. The oval piece of the intestine is now sutured with the skin of the abdominal wound and the wound is closed in the usual manner. The supporting sutures must be taken out one day or two days after the operation.

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J.6.2 Gall bladder motility

J.6.2.1 Activity on isolated gall-bladder strips from guinea pigs

PURPOSE AND RATIONALE

Effects on the smooth musculature of the gall bladder can be studied in isolated strips of gall bladder from guinea pigs or cats (Chowdhury et al. 1975; Fara and Erde 1978; Cabrini et al. 1995).

PROCEDURE

Guinea pigs of either sex weighing approximately 400 g are anesthetized with 3 g/kg urethane i.p. The gall bladder is removed and cut into longitudinal strips 10×3 mm. The strips are suspended in a Krebs solution bath between a stationary hook and an isometric strain gauge. Tension is recorded on a polygraph. The bath is maintained at 37 °C and aerated with a gas mixture of 95% O_2 and 5% CO_2. After a half-hour stabilization period, test doses of acetylcholine (1 mg/ml) are added to determine viability of the preparation. Dose-response curves for the muscle strips are obtained by introducing one dose of the stimulating agent and waiting until the maximal response to that dose is reached (usually 5–15 min). The bath is then rinsed three times and 15 min allowed before a new dose is tested.

EVALUATION

Concentrations for the maximal response and ED_{50} values are calculated.

MODIFICATIONS OF THE METHOD

Eltze et al. (1997) found that contractions of the isolated guinea-pig gallbladder elicited by muscarinic stimuli are mediated by functional muscarinic M_3 receptors.

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Fara JW, Erde SM (1978) Comparison of *in vivo* and *in vitro* responses to sulfated and non-sulfated ceruletide. Eur J Pharmacol 47:359–363

J.6.2.2 Gallbladder motility in dogs

PURPOSE AND RATIONALE

Gall bladder motility can be measured in anesthetized dogs with intraluminal manometry.

PROCEDURE

Beagle dogs weighing 15–18 kg are fasted for 16 h prior to the experiment. Median laparatomy is performed under pentobarbital anesthesia (25 mg/kg i.v.). An incision is made in the bile bladder and a 2 mm diameter polyethylene catheter introduced. The catheter is advanced as far as possible to the neck of the gall bladder and tied. The pressure in the interior of the gall bladder is recorded on a pen recorder via a Statham pressure transducer (P 23 BB) and a frequency measuring bridge.

EVALUATION

Cholecystokinin increases dose-dependent the intraluminal pressure. The preparation can be used for evaluation of cholecystokinin or cholecystokinin-like activity.

MODIFICATIONS OF THE METHOD

Ryan and Cohen (1976) used adult opossums (*Didelphis viginiana*) of either sex, weighing 2.5–3.0 kg.

REFERENCES

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J.6.2.3 Cholecystokinin activity (isolated gallbladder or intestine)

PURPOSE AND RATIONALE

A sensitive bioassay for cholecystokinin utilizing strips of rabbit gall bladder has been described by Amer and Becvar (1969). Segments of ileum and colon have been used by Paton and Zar (1968) and by Zetler (1984).

PROCEDURE

Male albino rabbits weighing 1.5–2.5 kg are fasted 18 h prior to the experiment. The animals are sacrificed by cervical dislocation and the liver is quickly dissected out. The gallbladder is teased away from the liver and immediately placed in Locke-Ringer solution. The biliary contents are emptied and the gall bladder is cut spirally (right-handed spiral starting from the bile duct) into a strip of muscle tissue 30–40 mm in length and about 5 mm wide. The muscle strip is placed in an organ bath containing Locke-Ringer solution at 37 °C continuously bubbled with carbogen. The initial tension of the muscle is adjusted to 0.5 g. The bath fluid is changed every 30 min. The test compound and the standard (range 5–20 mIDU (Ivy dog units) are added alternatively. The maximum of contractions is usually reached after 5 min. Then, the bath fluid is changed. The next dose is applied when the tension has achieved again the starting value.

EVALUATION

The maximum of contraction is taken as endpoint. Using at least two doses of test compound and standard, parallel line assays can be carried out and potencies ratios can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

This bioassay has the advantage to be sensitive and less time consuming than *in vivo* assays, such as measuring gall bladder motility in the anesthetized dog (see J.6.2.2). However, the assay is not specific for cholecystokinin, since also gastrin and gastrin analogues cause contractions of the isolated bile bladder. This assay allows also to calculate potency ratios of different gastrin analogues.

MODIFICATIONS OF THE METHOD

The whole gallbladder of the mouse or a strip of guinea pig gallbladder is used for evaluation of cholecystokinin-like peptides, ceruletide, ceruletide analogues, and cholecystokinin octapeptide by Zetler (1979, 1984)

Paton and Zar (1968), Zetler (1984), Chang and Lotti (1986), Barthol and Holzer (1987) used isolated segments of ileum and colon from guinea pigs for determination of CCK-like activity.

Makovec et al. (1986), Tachibana et al. (1996) studied the *in vivo* activity of derivatives of CCK in emptying the gallbladder in mice.

Henke et al. (1997) tested CCK-A agonists in the isolated guinea pig gall bladder.

Singh et al. (1995) used gall bladder strips from guinea pigs to evaluate CCK receptor antagonists.

Fukamizu et al. (1998) tested the effect of a cholecystokinin-A receptor antagonist against CCK-8 induced contractions in isolated smooth muscle fibers of gall bladders and longitudinal fibers of ileum from guinea pigs.

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J.6.3 Sphincter Oddi function

J.6.3.1 Relaxation of sphincter of Oddi *in vitro*

PURPOSE AND RATIONALE

The integrity of the relaxation function the sphincter of Oddi is a prerequisite for normal delivery of bile into the duodenum. Sphincter of Oddi relaxation is mainly executed by non-adrenergic, non-cholinergic (NANC) nerves that are essentially nitrergic in several species including guinea pigs (Pauletzki et al. 1993) and rabbits (Lonovics et al. 1994).

PROCEDURE

Biliary sphincter of Oddi muscle rings of approximately 6 mm length from adult male New Zealand white rabbits weighing form 3 500–4 000 g pretreated with various drugs or diet are prepared. The papilla Vater is eliminated and the ampullary part of the muscle rings of approximately 3 mm length are mounted horizontally on two small L-shaped glass hooks one of which is connected to a force transducer attached to a polygraph for measurement and recording of isometric tension. The experiments are carried out in an organ bath (5 ml) containing Krebs bicarbonate buffer which is maintained at 37 °C and aerated continuously with carbogen. The initial tension is set at 10 mN and the rings are allowed to equilibrate over 1 h during which period the sphincters develop characteristic 14–19 per min rhythmic contractions. Atropine (1 μM) and guanethidine (4 μM) are continuously present (NANC solution). Changes in isometric tension in response to two consecutive trains of impulses of electrical field stimulation (40 stimuli, 50 V, 0.1 ms and 20 Hz) are then studied.

EVALUATION

The data representing changes in isometric tension expressed as means ± standard deviation are evaluated by means of analysis of variance (ANOVA) followed by a modified Student's *t*-test for multiple comparisons according to Bonferroni's method. Changes are considered statistically significant at P-values smaller than 0.05.

MODIFICATIONS OF THE METHOD

In addition to studies with the isolated sphincter of Oddi of rabbits (Slivka et al. 1994; Sari et al. 1998; Jia and Stamler 1999) and guinea pigs (Harrington et al. 1992; Gocer et al. 1995; Lu et al. 1997), several pharmacological studies were reported with the isolated sphincter of Oddi of **opossum** (Perodi et al. 1990; Allescher et al. 1993), and of the **Australian brush-tailed possum** (*Trichosurus vulpecula*) (Baker et al. 1992, 1996).

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J.6.3.2 Function of sphincter of Oddi *in vivo*

PURPOSE AND RATIONALE

Dogs were used for *in vivo* studies of the function of the sphincter of Oddi (Sarles 1986; Pozo et al. 1990; Matsumara et al. 1991; Kobayashi et al. 1994; Wang et al. 1998). Shima et al. (1998) recorded the spontaneous motility and the response to cerulein on the canine sphincter of Oddi using a constant-perfusion technique.

PROCEDURE

Mongrel dogs weighing 7–9 kg fasted overnight are anesthetized with 25 mg/kg intravenous pentobarbital sodium and are maintained under adequate anesthesia with 12.5 mg/kg intravenous pentobarbital sodium as required. Systemic blood pressure is monitored through a catheter placed into the femoral artery. A femoral vein is cannulated and used for systemic administration of Ringer's solution and drugs.

After an upper median laparatomy, a small longitudinal incision is made in the common bile duct. Two catheters (outer diameter 2.0 mm) are cannulated and tied in the bile duct to avoid any leaks and the occlusion of the orifice of the catheters. One is distally placed at 5 mm proximal to the choledochoduodenal junction and is used to perfuse the sphincter of Oddi with saline at a rate of 0.12 ml/min using an infusion pump. The other is proximally placed and used to siphon off the hepatic bile. Pressure changes are recorded on a polygraph through a pressure transducer which is placed between the infusion pump and the catheter.

EVALUATION

To evaluate the effects of intravenously administered drugs on the sphincter of Oddi, a motility index is calculated by measuring the square between the zero line and the trace of the sphincter of Oddi pressure changes per minute.

Results are expressed as means ±SEM; n is the number of independent observations of different animals. The paired and unpaired t-tests are used for statistical analysis. $P < 0.05$ is considered significant.

MODIFICATIONS OF THE METHOD

Thune et al. (1992, 1995) studied simultaneously the flow resistances in the common bile duct and main pancreatic duct sphincters in anesthetized **cats** using a perfusion technique.

Elbrønd et al. (1994) prepared the sphincter of Oddi and duodenum in anesthetized **rabbits** with perfused catheters and bipolar electrodes. Increasing, successive doses of cholecystokinin were administered intravenously every 15th min. The digitized recordings were scored on a computer in control and stimulatory cholecystokinin sequences.

Further studies in **rabbits** were performed by Nakamura (1996), Chiu et al. (1998).

Opossums were used by Calabuig et al. (1990), Hanyu et al. (1990),Cullen et al. (1996), Herrmann et al. (1999) to study the function of the sphincter of Oddi.

Several authors used the **Australian brush-tailed possum** (*Trichosurus vulpecula*) for studies of the function of the sphincter of Oddi *in vivo* (Baker et al. 1990; Saccone et al. 1992; Cox et al. 1998a,b; Huang et al. 1998).

The **prairie dog** was used by several authors to study the function of the sphincter of Oddi (Ahrend et al. 1992; Kaufman et al. 1993; Thierney et al. 1994).

Pasricha et al. (1995) reported a model in **pigs** for endoscopic biliary manometry, similar in technique to the procedure in humans.

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J.7 Pancreatic function

J.7.0.1 Acute pancreatic fistula in rats

PURPOSE AND RATIONALE

The effect of exogenous hormones, e.g. secretin, or other drugs on pancreas secretion can be measured in rats with acute pancreas fistula.

PROCEDURE

Male Sprague Dawley rats weighing 150–200 g are used. Eighteen hours prior to the experiment food is withdrawn with free access to water. Groups of 4–5 rats are used for each dose of drug evaluation, the control group consists of at least 7 rats. The animals are anesthetized with 5 ml 25% urethane solution i.m. and the is trachea cannulated. The abdomen is opened by a mid-line incision and the pylorus ligated. The proximal part of the bile duct is ligated near the hepatic porta. The bile is drained via a thin polyethylene tube into the duodenum. The distal part of the bile duct with the orifices of pancreatic ducts is cannulated with another thin polyethylene tube. The pancreatic juice is collected in graduated microvessels. After a pre-test period of 60 min, the test compounds are applied intravenously or intraduodenally.

EVALUATION

The secretion after injection of the test compound is compared with the pre-test values. Secretin increases pancreatic secretion volume in a dose-dependent manner. Activity ratios for unknown preparations can be calculated by 2 + 2 assays in comparison with the international standard.

MODIFICATIONS OF THE METHOD

Guan et al. (1990) inserted two separate cannulae for bile and pancreatic juice to Wistar **rats** under methoxyfluorane anesthesia. Both fluids were returned to the intestine. Placing the rats in modified Bollman-type restraint cages, experiments could be performed after a few days in conscious animals.

Ito et al. (1994) studied the inhibition of CCK-8-induced pancreatic amylase secretion by a cholecystokinin type-A receptor antagonist in rats.

Niederau et al. (1989) compared the effects of CCK receptor antagonists on rat pancreatic secretion *in vivo*. Output of amylase in pancreatico-biliary secretion was measured after various doses of caerulein. The effects of high caerulein doses were dose-dependent inhibited by CCK-antagonists.

Alvarez and Lopez (1989) studied the effect of alloxan diabetes on exocrine pancreatic secretion in the anesthetized **rabbit**. After a 14–15 h fasting period, but with free access to water, rabbits weighing about 2.0 kg are anesthetized by intravenous injection of 1.0 g/kg urethane. After tracheotomy, a median laparatomy is performed, the main pancreatic duct is exposed and cannulated near its entrance to the duodenum following ligation of the pylorus and cannulation of the bile duct for deviation of bile to the exterior.

Kim et al. (1993) studied the effect of [(CH_2NH)4,5] secretin on pancreatic exocrine secretion in **guinea pigs** and rats using an acute pancreatic fistula preparation.

Niederau et al. (1990), Tachibana et al. (1996) determined pancreatic exocrine secretion in **mice**. Because the cannulation of mouse pancreatic duct is not possible for technical reasons, the amount of amylase was determined *in vivo*. Five min after i.v. administration of test drugs, mice were sacrificed and a 5 cm-duodenal loop was removed. The duodenal contents were washed out with 1.0 ml ice-cold saline and collected for amylase activity.

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J.7.0.2 Exocrine secretion of isolated pancreas

PURPOSE AND RATIONALE

Procedures for isolation and perfusion of rat pancreas in order to record membrane potential and effective membrane resistance during the collection of perfusates and for simultaneous measuring of flow of pancreatic juice were described by Kanno (1972), Kanno and Saito (1976), Kanno et al. (1976), Saito et al. (1980). Similar techniques to measure exocrine secretion of the isolated pancreas have been used by other groups (Penhos et al. 1969; Mann and Norman 1984; Trimble et al. 1985; Norman and Mann 1986, 1987; Norman et al. 1989; Park et al. 1993).

PROCEDURE

Male Sprague Dawley rats weighing about 250 g are fasted for 24 h before the experiments but are allowed to drink water. Under ether anesthesia and after cannulation, the vascular system and the common duct are separately perfused. The rectum and the transverse colon are separated from the pancreas following ligation and sectioning of their vascular supply. Perfusion is performed through the hepatic end of the common duct with the outlet at the duodenal end. The **flushing preparation** is used when continuously recording the transmembrane potential and, in this preparation, the common duct is flushed at a constant pressure of 5 cm H_2O following cannulation of both ends with stainless steel tubes. The **draining preparation** is used during the electrical measurements are made from as many acinar cells as possible in every 10 min. In the draining preparation, the hepatic end of the duct is ligated and the pancreatic juice collected from the duodenal end following cannulation with a stainless steel tube. In both preparations, the inlets of the vascular perfusion are the superior mesenteric artery and the coeliac artery, and the outlet is the portal vein. The rate of vascular flow is kept constant at 1 ml/min with the aid of a roller pump. The animal is then killed by cutting the carotid arteries and the perfusion begins. The spleen is removed after section of its vascular supply close to the hilum, care being taken to avoid interference with the supply from the splenic artery. The blood supplying stomach and liver is stopped by tying the hepatic artery and gastric arteries. The superior mesenteric vein and the descending branch of the superior mesenteric artery are then ligated. The mesentery with its embedded whole pancreas and the attached duodenum is then removed and mounted on a paraffin block in a lucite chamber containing 20 ml of standard Krebs-Henseleit solution. The contents of the duodenum is then drained with a polyethylene tube. The level of the standard Krebs-Henseleit solution in the bath is kept constant with a siphon. The temperature of the preparation and perfusing Krebs-Henseleit solution containing 8 vol% erythrocytes is maintained at 37 °C. The perfusion solution in the reservoir is continuously bubbled with 5% CO_2 in O_2.

The rate of flow of pancreatic juice is measured by attaching a calibrated tube made of silicone-rubber to the free end of the pancreatic duct cannula. Every

10th min the tube is replaced and the rate of flow of pancreatic juice is noted. The sample is diluted with Krebs-Henseleit solution up to 100 ml and the amount of amylase assessed.

Intracellular recordings are made from the pancreatic acinar cells by manually advancing KCl-filled microelectrodes under direct visual control. The microelectrode is connected to the probe of a solid state preamplifier. Resting potentials are observed at the screen of a cathode ray oscilloscope and simultaneously recorded on a direct visual oscillograph.

Measurements are made every 10 min for half an hour as base level, followed by measurements every 10 min during infusion of drug for one hour.

EVALUATION

All results are expressed as mean ±SEM. Statistical analysis is performed using Student's *t*-test.

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J.7.0.3 Chronic pancreatic fistula in rats

PURPOSE AND RATIONALE

Suguiyama et al. (1996) described the preparation of a chronic pancreatic fistula in the WBN/Kob rat, a strain which develops spontaneously chronic pancreatitis (Ohashi et al. 1990; see also J.7.0.14).

PROCEDURE

Male WBN/Kob rats at an age of 2 months are used. Before surgery, the rats have free access to chow and water. After an 18-h fast, laparatomy is performed through a midline incision under general anesthesia with ether. A polyethylene catheter (inside diameter 0.28 mm; outside diameter 0.61 mm, length 30 cm), as an external pancreatic fistula, is inserted into the common biliary pancreatic duct immediately proximal to the ampulla of Vater. The bile duct is ligated proximally to the pancreas. Another catheter is introduced into the bile duct distal to the confluence of the hepatic ducts, and the opposite end of the catheter is inserted into the duodenum near the ampulla through the stomach to drain the bile into the intestine. A third catheter is inserted in the duodenum through the stomach to return pancreatic juice. A fourth catheter is placed in the femoral vein, and 0.15 M sodium chloride is continuously administered at a flow rate of 1 ml/h with a syringe pump. All catheters are brought into the abdominal cavity or the femoral region through a subcutaneous tunnel starting in the middle portion of the tail. The rat is then placed prone in a cage with the proximal portion of the tail fixed to a side wall of the cage. When awakened, the rat is allowed access to food and water ad libitum. Pancreatic juice is collected and continually re-circulated with a syringe pump every 6 h until the secretion test.

The secretion test is started 3 days after surgery. After a 12-h fast, pancreatic juice is collected every 30 min in a plastic syringe. The juice volume is determined by weighing. A 50-µl sample is taken for the analysis of juice composition, and the remaining juice is returned to the duodenum for the next 30-min collection period.

After a 30-min basal period, the test compound is administered in graded doses, each dose for 30 min.

The bicarbonate concentration of pancreatic juice is measured with a microgasometer (Natelson 1958) in a sample size of 10 µl. Protein concentration is determined by measuring optical density at 280 nm with purified bovine trypsinogen as standard.

EVALUATION

All values are presented as mean ±SEM. Results are analyzed by means of the Wilcoxon test. Differences are considered significant at $p < 0.05$.

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J.7.0.4 Acute pancreatic fistula in dogs

PURPOSE AND RATIONALE

The effect of exogenous hormones, e.g. secretin or gastrin, or of vagal stimulation, on pancreatic secretion can be measured in dogs with acute pancreas fistulas.

PROCEDURE

Beagle dogs of either sex weighing 12–20 kg are used. The animals are fasted for a 24-h period and then anesthetized with 25 mg/kg sodium pentobarbital i.v. After opening the abdomen along the mid-line, the pyloric sphincter is ligated and the common bile duct cannulated to prevent the entry of acid chyme and bile into the duodenum. The bile is allowed to drain. The pancreas is gently exposed and the major pancreatic duct ligated. A polyethylene tube of 2 mm diameter is inserted into the minor pancreatic duct for collection of the secretion. The left femoral vein is cannulated for continuous infusion or i.v. injection. The pancreatic juice is collected in an ice-bath in a special tapered tube with fine calibrations for measuring volumes of less than 1 ml.

At the end of each collection period, the volume is recorded, and the bicarbonate content determined titrimetrically. Furthermore, pancreatic enzymes, such as amylase, are determined in the samples. Determination of protein concentrations in the pancreatic juice can be used as end-point since the total protein concentration is proportional to the individual enzymes (Keller et al. 1958). In a pretest period of 10 min, samples are collected every 2 min. Then, the test compound is injected intravenously and the pancreatic juice is collected every 2 min.

EVALUATION

The secretion after injection of the test compound is compared with the pre-test values. Secretin increases pancreatic volume and bicarbonate secretion in a dose-dependent manner. Activity ratios for unknown preparations can be calculated by 2 + 2 points assays in comparison with the international standard.

Moreover, the model can be used for standardization of gastrin analogues.

MODIFICATIONS OF THE METHOD

Glad et al. (1996) tested the influence of gastrin-releasing peptide on acid-induced secretin release and pancreatobiliary and duodenal bicarbonate secretion in Danish country strain **pigs** weighing between 22 and 30 kg. The animals, starved overnight with free access to water, were premedicated with 4 mg/kg i.m azaperone, and with 5 mg/kg i.p. metomidate. After 20 min a cannula was placed in an ear vein, and 5–10 mg/kg metomidate was given i.v. followed by intubation and artificial respiration with 50% O_2 and 50% N_2O. Anesthesia was maintained with an intravenous bolus infusion of 0.53% α-chloralose.

Both external jugular veins were cannulated for infusion of saline or drugs. A femoral artery was cannulated for withdrawal of blood samples and recording of blood pressure. After laparatomy the cystic duct was ligated, and the common hepatic duct and the pancreatic duct were catheterized. The duodenal segment was defined as extending from the pylorus to the ligament of Treitz. A Foley catheter was passed through the pylorus into the proximal part of the duodenum and inflated. Distal to the pylorus the pancreaticoduodenal arteries, veins and nerves were dissected, and a double ligature was passed under these structures and tied around the duodenum. At the ligament of Treitz an inflated Foley catheter was placed in the distal part of the duodenum and tied with a suture around the duodenum. A catheter was placed through a splenic branch of the left gastroepiploic vein and advanced through the lienal vein to the portal vein.

The flow of pancreatic juice and bile was tested before and after the experiment by means of an intravenous bolus of 5 pmol/kg secretin. Before the experiment the duodenum was continuously perfused at a rate of 2 ml/min for 435 min with isotonic saline containing phenol red (10 mg/l) as a marker. After drug treatment (intravenous infusion of gastrin-releasing peptide or duodenal HCl perfusion) pancreatic and hepatic secretions were collected in 15-min periods and the volumes determined by weighing. Duodenal effluents were collected in 15-min periods and phenol red concentrations determined spectrophotometrically. Blood sampled were withdrawn for determination of secretin by radioimmunoassay.

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J.7.0.5 Chronic pancreatic fistula in dogs

PURPOSE AND RATIONALE

Herrera et al. (1968) described a special cannula which can be used to obtain pancreatic juice or bile from a duodenal pouch after appropriate surgical procedures (Preshaw and Grossman 1965).

PROCEDURE

Male Beagle dogs weighing 15–20 kg are used. The abdomen is opened through a midline epigastric incision under barbiturate anesthesia. The duodenum is mobilized at the pyloric and jejunal ends, and a 5-cm duodenal segment containing the main pancreatic duct is isolated. The proximal level of the duodenal section lies immediately distal to the opening of the common bile duct and the distal level of section lies 2.5 cm distal to the main pancreatic duct. The distal stoma of the duodenum is closed and continuity restored by end-to-side duodeno-jejunostomy. The duodenal pouch is closed at both ends.

The cannula to be inserted is made of stainless steel and consists of 3 parts. The main casement measures 10.5 cm in length, with an external diameter of 1.0 cm. The internal end is flanged, and 1.5 cm from this point there is a short lateral limb, also 1.5 cm in length. The lateral limb is also flanged but, in addition, possesses a small V-shaped defect to facilitate insertion into the pouch.

When not in use, the cannula is sealed from the exterior by inserting a threaded plug which allows pancreatic juice to enter the duodenum in the normal manner. For collection of juice this plug is removed and a long obturator is inserted. The latter effectively isolates the pancreatic secretion from other duodenal contents. A similar hollow obturator is reserved for use when duodenal perfusion is studied, the obturator being connected via a plastic tube to the irrigating fluid.

Through a small antimesenteric incision in the duodenal pouch, the lateral limb of the cannula is inserted; the V-shaped defect in the flange facilitates entry into the pouch. A purse string secures the cannula in position. The defunctioned loop of duodenum is then brought anterior to the pancreas, and the remaining limb of the cannula inserted through a small duodenotomy and secured further by a purse-string suture. The whole system is then generously wrapped in omentum and the cannula exteriorized through a stab incision toward the flank. An external collar stabilizes the cannula.

The cannula is left open to drain blood and secretions for 24 h postoperatively, after which time the plug is inserted. Physiologic saline is administered subcutaneously for a period of 3 days, after which the animals are permitted to drink water. Checking the cannula daily is advisable to ensure that the plug remains tight. The animals receive normal kennel food and water ad libitum.

The dogs are allowed at least 2 weeks to recover. Eighteen hours prior to the experiment food is withdrawn but water allowed ad libitum. The long obturator is inserted and pancreatic juice collected for 15 min periods. After 1 h pretest time, the test compound is given either orally or intravenously.

EVALUATION

Secretion of pancreas juice is measured at 15 min intervals and volume and enzyme content determined. The values are compared with pretest data.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described details of the technique as recommended by Pavlov (1902) as well as his own modification.

Konturek et al. (1976, 1984) performed experiments with chronic gastric fistulas in **cats** as well as in **dogs** to compare the species-specific activities of vasoactive intestinal peptide and secretin in stimulation of pancreatic secretion.

Ninomiya et al. (1998) studied the effects of a cholecystokinin A receptor antagonist on pancreatic exocrine secretion stimulated by exogenously administered CCK-8 in conscious dogs with chronic pancreatic fistula.

Garvin et al. (1993) described distal pancreatectomy with autotransplantation and pancreaticocystostomy in dogs.

Kuruda et al. (1995) developed a new technique in dogs for pancreatico-gastrointestinal anastomosis that consists of pancreatectomy using the ultrasonic dissector and implantation of the pancreatic duct into the gastrointestinal tract without suturing the pancreatic parenchyma.

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J.7.0.6 Somatostatin activity

PURPOSE AND RATIONALE

Somatostatin is a neuropeptide, also called somatotrophin release inhibiting factor (SRIF or SRIF-14), which occurs not only in the brain, but also in a variety of peripheral tissues. Two other related peptides somatostatin-28 (SRIF-28) and cortistatin (Vasilaki et al. 1999), also occur naturally (Humphrey 1998). Somatostatin acts via G-protein coupled receptors of which five subtypes are cloned (Hoyer 1998).

Somatostatin analogues and antagonists have been tried for a variety of indications (Bass et al. 1996; Coy and Taylor 1996; Papageorgiou and Borer 1996; Rohrer et al. 1998; Yang et al. 1998). The long-acting octapeptide somatostatin analog, octreotide, has been studied thoroughly (Ambler et al. 1996; Danesi and Del Tacca 1996; Hoffmann et al. 1996; Paran et al. 1996) and gained clinical use as gastric antisecretory agent.

Somatostatin inhibits basal pancreatic secretion as well as secretion stimulated by food, cholecystokinin, and secretin. Inhibition of pancreatic secretion can be used as bioassay to compare synthetic analogues with the original somatostatin.

PROCEDURE

Male Wistar rats weighing 300–400 g are anesthetized with methoxyfluorane and prepared with silastic cannulae. Two cannulae drain pancreatic juice and bile separately, one cannula is inserted into the duodenum for return of bile and pancreatic juice to the intestine and for intestinal infusion. Another cannula is located in the abdominal cavity and a fifth cannula into the right jugular vein for drug infusion. After surgery, the rats are placed into modified Bollman-type restrain cages. The rats have free access to food and water. During recovery and between the experiments, pancreatic juice and bile are collected and continuously returned to the intestine by a servo-system consisting of a collection tube in a liquid-level photodetector coupled to a peristaltic pump.

Experiments are performed on the third to eighth days postoperatively. The rats are assigned to treatment groups on a random basis and, treatment days are equally divided among the third to eighth postoperative days. The rats are fasted overnight before each experiment. Bile and pancreatic juice are collected separately every 30 min; the volume of pancreatic juice is measured, and 10 ml is taken for protein assay. Different doses of the test drug and the standard are given as continuous i.v. infusion over a period of 2 h.

EVALUATION

Inhibition of pancreatic fluid and protein output is calculated compared to control infusion and expressed as percentage. From dose-response curves activity ratios can be calculated.

MODIFICATIONS OF THE METHOD

Konturek et al. (1985) and Susini et al. (1980) studied the effect of somatostatin analogues on pancreas secretion in dogs.

Cai et al. (1986) reported on the biological activity of octapeptide analogs of somatostatin assaying GH concentrations in blood samples of anesthetized rats by RIA and studying the inhibition of gastric secretion on dogs.

Biological actions of prosomatostatin have been described by Meyers et al. (1980) using *in vitro* and *in vivo* GH bioassays.

Taylor et al. (1996) employed the technique of cytosensor microphysiometry for real-time evaluation of somatostatin subtype receptor activity in CHO-K_1 cells stably expressing the human sst_2 receptors.

Cytotoxic analogs of somatostatin have been synthesized and evaluated (Nagy et al. 1998).

Gilon et al. (1998) studied a synthetic receptor 5-selective somatostatin analogue *in vivo* in rats. The compound inhibited bombesin- and caerulein-induced amylase and lipase release from the pancreas without inhibiting growth hormone or glucagon release.

Jeandel et al. (1998) described the effects of two somatostatin variants on receptor binding, adenyl cyclase activity and growth hormone release from the frog pituitary.

Hofland et al. (1994) determined relative potencies of somatostatin analogs on the inhibition of growth hormone release by cultured human endocrine tumor cells and normal rat anterior pituitary cells.

Somatostatin analogs labeled with ^{99m}technetium, ^{111}indium, and ^{90}yttrium were developed for tumor diagnosis and therapy (O'Byrne and Carney 1996; Pearson et al. 1996; Stolz et al. 1996, 1998; Thakur et al. 1996).

Radioimmunoassays for somatostatin have been developed (Arimura et al. 1975; Gerich et al. 1979; Patel and Reichlin 1979; Patel 1984) and are available as commercial kits.

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J.7.0.7 Receptor binding for somatostatin

GENERAL CONSIDERATIONS

A family of five somatostatin receptor subtypes from various species including man has been described (Yamada et al. 1992; Reisine and Bell 1995; Hoyer et al. 1995; Bruns et al. 1996; Liapakis et al. 1996; Patel et al. 1996; Nilsson and Folkesson 1997; Patel 1997; Pscherer et al. 1996; Shimon et al. 1997; Humphrey et al. 1998). There are several endogenous ligands, such as somatostatin-14, somatostatin-28 and the corticostatins (Meyerhoff et al. 1992; De Lecea 1996; Fukusumo 1997). A number of synthetic peptide analogues have been developed and their relative affinities at human recombinant receptors in radioligand receptor studies have been established (Raynor et al. 1992; Bass et al. 1996; Coy and Taylor 1996; Pearson et al. 1996; Piwko et al. 1996, 1997a,b).

Greenmman and Melmed (1994) evaluated the expression of three somatostatin receptor subtypes (SSTR3, SSTR4, and SSTR5) in pituitary tumor specimens. SSTR3 expression was studied by reverse transcription coupled to polymerase chain reaction, whereas SSTR4 and SSTR5 expression was determined by ribonuclease protection assay.

Somatostatin occurs not only in the hypothalamus and in the gut, but also in several other organs. Somatostatin$_1$ receptors in the nucleus accumbens have been found to mediate the stimulatory effect of somatostatin on locomotor activity in rats (Raynor et al. 1993a). Molecular cloning and expression of a pituitary somatostatin receptor with preferential affinity for somatostatin-28 has been described by O'Carroll et al. (1992).

Cloning and expression of a mouse somatostatin receptor (SSTR 2B) has been reported by Vanetti et al. (1992). Pharmacological properties of two cloned somatostatin receptors have been described by Rens-Domiano et al. (1992).

Subtype-selective peptides have been identified by Raynor et al. (1993b). The somatostatin receptor, SSTR3, coupled to adenyl cyclase, has been cloned by Yasuda et al. (1992). Somatostatin receptors SSTR4 and SSTR5 have been cloned and characterized by Raynor et al. (1993c).

The somatostatin receptor SST_1 mediates inhibition of central neurons (Viollet et al. 1997).

The somatostatin receptor SST_2 inhibits growth hormone release (Raynor et al. 1993b), inhibits parietal cell function and ion secretion in rat distal colon (McKeen et al. 1996; Warhurst et al. 1996; Wyatt et al. 1996), and inhibits neurons in the rat locus coeruleus (Chessell et al. 1996). High basal gastric acid secretion has been found in somatostatin receptor subtype 2 knockout mice (Martinez et al. 1998). The cytoplasmatic tail of the somatostatin receptor SST_2 undergoes alternative splicing giving rise to two isoforms, SST_{2A} and SST_{2B} (Schulz et al. 1998).

The somatostatin receptor sst_3 mediates relaxation of isolated gastric smooth muscle cells (Gu et al. 1995).

The fourth human somatostatin receptor has been cloned and characterized by Rohrer et al. (1993).

The somatostatin receptor sst_5 may have a role in inhibition of hormone release from the pituitary (Tallent et al. 1996) and pancreas (Rossowski and Coy (1994) and mediates the antiproliferative effect of somatostatin in vascular smooth muscle (Lauder et al. 1997).

PROCEDURE

Crude membrane preparations are obtained by homogenizing (Polytron, setting 6, 15 s) tumor cell cultures

(e.g. human SCLC line NCI-H69, rat pancreatic tumor AR42J) or rat tissues (lung, pancreas, cerebral cortex) in ice-cold 50 mM Tris-HCl and centrifuging twice at 39 000 *g* (10 min), with an intermediate resuspension in fresh buffer. The final pellets are resuspended in 10 mM Tris-HCl for assay. Aliquots of the membrane preparation are incubated for 25 min at 30 °C with [^{125}I-Tyr^{11}]SRIF (2 000 Ci/mmol, Amersham) in 50 mM HEPES (pH 7.4) containing bovine serum albumin (10 mg/ml; fraction V, Sigma), $MgCl_2$ (5 mM), Trasylol (200 KIU/ml), bacitracin (0.02 mg/ml), and phenyl-methyl-sulfonyl fluoride (0.02 mg/ml). The final assay volume is 0.3 ml. The incubations are terminated by rapid filtration through Whatman GF/C filters (presoaked in 0.3% polyethylenimine) under reduced pressure. Each tube and filter is then washed three times with 5 ml aliquots of ice-cold buffer. Specific binding is defined as the total [^{125}I-Tyr^{11}]SRIF bound minus that bound in the presence of 200 nM unlabeled SRIF.

EVALUATION

The binding parameters are calculated from the experimental data by non-linear least-squares regression analysis using an appropriate computer program.

CRITICAL ASSESSMENT OF THE METHOD

Somatostatin occurs in many organs and has more activities than anticipated at its discovery. Therefore, the occurrence of a family of receptors rather than a single receptor is not surprising.

MODIFICATIONS OF THE METHOD

Receptor scintigraphy with a radioiodinated somatostatin analogue has been described by Bakker et al. (1990).

The tissue-selective binding of somatostatin-14 and somatostatin-28 in rat brain was studied by Srikant et al. (1990).

Feniuk et al. (1993) characterized somatostatin receptors in guinea-pig isolated ileum, vas deferens and right atrium.

Yang et al(1998) reported synthesis and biological activities of potent peptidomimetics selective for somatostatin receptor subtype 2 by testing inhibition of forskolin-stimulated cAMP accumulation in a mouse cell line that contains a cAMP response element fused to the *E. coli* β-galactosidase gene and by measuring growth hormone release from primary cultures of rat anterior pituitary cells.

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J.7.0.8 Secretin activity

PURPOSE AND RATIONALE

Synthetic derivatives of secretin can be tested for activity against the standard using the bioassay of a chronic pancreatic fistula in dogs. Moreover, stability of secretin preparations has to be checked when using this bioassay.

PROCEDURE

Dogs with a chronic pancreatic fistula prepared according to Preshaw and Grossman (1965), Herrera et al. (1968) (see J.6.1.4) are used. Eighteen hours prior to the experiment food is withdrawn but water allowed ad libitum. Pancreatic juice is collected for 15 min periods. After 1 h pretest time, a test dose of 0.2 KE/kg Karolinska-secretin is given intravenously. In one hour intervals the test preparation or the standard is given alternatively in logarithmic doses between 0.1 and 1.6 KE/kg intravenously. The volume of pancreas juice secreted during 30 min after injection is used as parameter.

EVALUATION

Using at least two doses of test compound and standard, parallel line assays can be carried out and potencies can be calculated. Each of 4 animals receives two doses test compound and two doses standard, the application scheduled according to a Latin square. Potency ratios with confidence limits are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method is time consuming and needs dogs with chronic fistula. Nevertheless, for final evaluation of a test compound this bioassay can not be avoided.

MODIFICATIONS OF THE METHOD

The standardization of secretin has been described as early as 1952 by Burn et al. using anesthetized **cats**. After laparatomy the pylorus and first duodenal loop are brought outside and turned to the animal's left side. The bile duct is identified by gently squeezing the bile-bladder. The pancreatic duct usually enters the duodenum about 2 mm below the entry of the bile-duct. Starting about 2 cm below the bile duct, the pancreas is separated from the duodenum by blunt dissection, the blood vessels being tied and divided where necessary. When the pancreatic duct is reached, two silk ligatures are placed around and a length of 5 mm is dissected. A dose of secretin is injected intravenously and one of the ligatures is then tied as near the duodenum as possible. The duct is then cut and a flow of juice is usually visible. A small cannula is tied into the duct and attached to a rubber tube and L-piece whose position is adjusted so that the flow of juice continues. The cannula and the L-piece are clamped in position and the abdomen is closed.

The relation between the dose injected and the number of drops of pancreatic juice secreted is almost linear, but the slope differs in different cats. In making a comparison between two preparations, the dose-response relation is first determined for the standard preparation by administration of three or more different doses. A dose of the test preparation is then injected and the effect compared with the curve of the standard. Further doses of the test preparation and comparison of the dose-response curves allow the calculation of the relative potency.

Izzo et al. (1989) studied the internalization of labelled secretin into isolated pancreatic acinar cells of **rats**.

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J.7.0.9 Receptor binding for secretin

PURPOSE AND RATIONALE

Secretin receptor antagonists, such as reduced peptide bond pseudopeptide analogues of secretin, inhibit the binding of ^{125}I-secretin to secretin receptors in pancreatic acini (Haffar et al. 1991).

Radioimmunoassays for secretin have been developed (Boden and Wilson 1979; Chang and Chey 1980) and are available as commercial kits.

PROCEDURE

Dispersed acini from guinea pig pancreas are prepared according to Peikin et al. (1978) and Jensen et al. (1982). After sacrifice, the pancreas of a guinea pig is immediately removed and trimmed of fat and mesentery. A suspension of dispersed acini is prepared by injecting the pancreas 5 times with 5 ml of digestion solution composed of standard incubation solution plus purified collagenase (0.12 mg/ml). The pancreas is in-

cubated for 4 sequential 10-min periods at 37 °C in a Dubnoff incubator at 160 oscillations per min. The gas phase is 100% O_2. The digestion solution is discarded, and the tissue is washed 3 times with 5 ml of standard incubation solution. The tissue is dispersed by passing it 5 times through each of a series of siliconized glass pipettes of decreasing bore (3, 1, and 0.5 mm). Large fragments and the duct system are discarded. The suspension of dispersed acini is layered over standard incubation solution containing 4% albumin and centrifuged at 800 *g*. The supernatant is discarded, and the acini are washed twice with standard incubation solution containing 4% albumin.

^{125}I-Secretin and ^{125}I-labeled analogues are prepared using chloramine T and a method described by Chang and Chey (1980), Jensen et al. (1983), Zhou et al. (1989). They are separated from ^{125}I using a disposable C_{18} cartridge (Sep-Pak) and then separated from unlabeled peptide with reverse phase HPLC using a 4.6-mm × 25-cm column of mBondapak C_{18}. The column is eluated with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (v/v) from 0 to 54% acetonitrile in 60 min using a flow rate of 1.0 ml/min.

For binding of ^{125}I-secretin incubations are performed containing 0.25 ml of cell suspension of pancreatic acini (one pancreas in 10 ml of standard incubation buffer) and 50 pM ^{125}I-secretin with or without 1 mM secretin. Nonsaturable binding of ^{125}I-secretin is measured as the amount of radioactivity associated with acini when the incubation contains 50 pM ^{125}I-secretin plus 1 mM secretin. Secretin antagonists are added in various concentrations. Saturable binding of ^{125}I-secretin is expressed as percentage of radioactivity bound in the absence of the antagonists.

EVALUATION

K_i values are calculated from percent of controls of bound ^{125}I-secretin using the equation:

$$K_i = [R / (R - 1)]\,[S\,B / (S + A)]$$

where R = the observed saturable binding of ^{125}I-secretin in the presence of the antagonist (B) expressed as a fraction of that obtained when B is not present. A is the concentration of ^{125}I-secretin (0.05 nM), S is the k_d determined by Scatchard analysis.

MODIFICATIONS OF THE METHOD

Bawab et al. (1991) characterized the down regulation of the ^{125}I-secretin binding sites and the associated desensitization of the secretion receptor-cAMP system in rat gastric glands.

Molecular cloning and expression of a cDNA encoding the secretin receptor in COS cells was reported by Ishihara et al. (1991).

Steiner et al. (1993) localized secretin receptors mediating rat stomach relaxation by autoradiography of frozen sections of the rat stomach with ^{125}I-labeled porcine secretin.

Ulrich et al. (1993) studied the intrinsic photoaffinity labeling of native and recombinant rat pancreatic secretin receptors.

Vilardaga et al. (1994) investigated the properties and regulation of the coupling to adenylate cyclase of secretin receptors stabile transfected in Chinese hamster ovary cells.

Molecular cloning, expression and functional characterization of a human secretin receptor was reported by Chow (1995), Patel et al. (1995), of the rabbit secretin receptor by Svoboda et al. (1996). The full-length human secretin receptor cDNA was subcloned into the mammalian expression vector pRc/CMV and expressed in cultured CHO cells (Ng et al. 1999). Intracellular cAMP accumulation of the stably transfected cells was measured by a radioimmunoassay, while the extracellular acidification rate was measured by the Cytosensor microphysiometer.

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J.7.0.10 Cholecystokinin activity (isolated rat pancreatic acini)

PURPOSE AND RATIONALE

Cholecystokinin (CCK) is one of the first discovered gastrointestinal hormones and one of the most abundant neuropeptides in the brain (Crawley and Corwin (1994). CCK is found in high concentrations in the mammalian brain and may be implicated in the neurobiology of anxiety and panic disorder (Bourin et al. 1996). Two types of CCK receptors have been identified: CCK-A receptors are mainly located in the periphery, but are also found in some areas of the CNS. CCK-B receptors are widely distributed in the brain. Major biological actions of CCK are the reduction of food intake and the induction of anxiety-related behavior. Inhibition of feeding is mainly mediated by the A-type receptors, whereas anxiety-like behavior is induced by stimulating B-type receptors (Fink et al. 1998).

Isolated pancreatic acini of rats can be used for evaluation of cholecystokinin activity of synthetic derivatives and for plasma CCK-like activity (Liddle et al. 1984; Höcker et al. 1990; Schmidt et al. 1991).

PROCEDURE

For plasma determinations, CCK or inhibitors are extracted from a plasma sample using PR-18 cartridges (Merck, Darmstadt, Germany). One ml plasma is diluted with 4 ml 0.1% trifluoroacetic acid and applied to a cartridge. After a wash (15 ml), CCK and inhibitor are eluted with acetonitrile/water (80 : 20, v/v) and lyophilized. Pancreatic acini are prepared from female Sprague-Dawley rats weighing 180–200 g, 1 to 2 weeks post ovarectomy, by enzymatic digestion of pancreas with collagenase (Jensen et al. 1982). Test compounds, or extracted material reconstituted with Krebs-Ringer HEPES buffer, or CCK standard is incubated with acini (final volume 0.25 ml). Lipase is measured with an autoanalyser (e.g., Hitachi type 705 or 805). Release is calculated as percent of the initial content determined in each incubation vial.

EVALUATION

CCK bioactivity is determined by comparison with a CCK-8 standard curve. Results are expressed as CCK-like bioactivity.

MODIFICATIONS OF THE METHOD

Amblard et al. (1998) evaluated cyclic cholecystokinin analogues for their ability to stimulate amylase secretion from isolated pancreatic acini.

Inhibition of CCK-8 induced release of amylase from pancreatic cells was used for measurement of CCK_A antagonism (Yamazaki et al. 1995; Akiyama et al. 1996; Patel et al. 1996; Taniguchi et al. 1996; Ballaz et al. 1997; Martin-Martinez et al. 1997).

Deyer et al. (1993) reported on acetylcholine and cholecystokinin induced acid extrusion in mouse isolated pancreatic acinar cells as measured by the microphysiometer. The microphysiometer continually measures the pH of the medium bathing a cell sample. EC_{50} values for the acidification rate were determined for CCK and CCK analogues. Dunlop et al. (1997) used the Cytosensor® microphysiometer to analyze the activity of cholecystokinin antagonists against the CCK-4-mediated response in hCCK-B CHO cells.

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J.7.0.11 Receptor binding of cholecystokinin

PURPOSE AND RATIONALE

Receptor binding assays for CCK have been described by several authors, such as Innis and Snyder (1980) for rat pancreas; Gaisano et al. (1989), Doi et al. (1990), Blevis et al. (1990), Maletínská et al. (1992) for isolated pancreatic acini; Saito et al. (1981), Chang et al. (1983) for guinea pig cerebral cortex, Praissman et al. (1983) for guinea pig mucosa, Van Dijk et al. (1984) for rat brain and pancreas; Steigerwalt et al. (1984), Chang and Lotti (1986) for gallbladder membranes; Kaufmann et al. (1993) for guinea-pig cerebral cortex and rat pancreas.

Using transfected COS cells, Talkad et al. (1994) identified three different states of the pancreatic CCK receptor, with the very-low-affinity state being the most abundant.

A review on cholecystokinin receptors was given by Wank (1995).

Radioimmunoassays for cholecystokinin have been developed (Harvey 1979) and are available as commercial kits.

PROCEDURE

Membranes of rat (Sprague Dawley) pancreas, guinea pig (Hartley) cerebral cortex, and bovine gallbladder are prepared by homogenization in 50–100 vol of 50 mM Tris-HCl (pH 7.4 at 37 °C) using a Polytron (Brinkman, PT 10, setting 4 for 10 s for pancreas or brain and maximal speed for bovine gallbladder). Homogenates are centrifuged at 50 000 *g* for 10 min, and the pellets are resuspended in the same buffer and centrifuged as described above. The resulting pellets are resuspended in 4 000, 80, and 25 ml of binding assay buffer for each g of original tissue wet weight of pancreas, brain, and gallbladder, respectively. ^{125}I-CCK-8 binding assay buffer contains 5 mM dithiothreitol, 50 mM Tris-HCl (pH 7.4 at 37 °C), 5 mM $MgCl_2$, 2 mg of bovine serum albumin, and bacitracin at 0.14 mg/ml for pancreas; 10 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, bacitracin at 0.25 mg/ml, and 130 mM NaCl (pH adjusted to 6.5 with NaOH) for brain; and 10 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, bacitracin at 0.25 mg/ml, soybean trypsin inhibitors at 0.2 mg/ml, and 130 mM NaCl (pH 6.5) for bovine gallbladder.

^{125}I Bolton-Hunter labeled CCK-8 (New England Nuclear) is used. Free and bound ^{125}I-CCK-8 are separated by filtration using Whatman G/F B glass fiber filters that are presoaked with 50 mM Tris-HCl (pH 7.4) containing bovine serum albumin (1 mg/ml). Immediately after the filtration, the filters are washed rapidly three times with 4 ml of Tris-HCl containing bovine serum albumin (0.1 mg/ml). Radioactivity is counted with a gamma counter, e.g. Beckman Instruments. Specific ^{125}I-CCK-8 binding is defined as the difference between total binding and nonspecific binding in the presence of 1 mM CCK.

EVALUATION

IC_{50} values are determined by regression analysis of displacement curves. Inhibitor constants (K_i) are calculated from the formula

$$K_i = I / (K_{d'} / K_{d\text{-}1})$$

where I is the concentration of the inhibitor, and K_d and $K_{d'}$ are the dissociation constants of ^{125}I-CCK-8 in the absence and presence of the inhibitor, respectively.

MODIFICATIONS OF THE METHOD

Subtypes of the cholecystokinin receptor, forming a cholecystokinin receptor family, have been described, such as CCK_A from pancreas and other parts of the gastrointestinal tract and in a few discrete brain regions (Kachur et al. 1991; Poirot et al. 1992; Wank et al. 1992a).

According to the IUPHAR Compendium of Receptor Characterization and Classification 1998; CCK_A and CCK_B receptors are designated as CCK_1 and CCK_2, respectively.

A survey on ligands for cholecystokinin receptors was given by Trivedi (1994).

Van der Bent et al. (1994) described molecular modeling of **CCK-A receptors**.

For CCK_A receptor binding assays (Fossa et al. 1997), the pancreas from a male Hartley guinea pig is dissected and placed in saline. Fatty tissue and blood vessels are dissected away and the tissue placed in 20 vol of buffer (50 mM Tris HCl, pH 7.4, 0.35 mg/ml bacitracin and 0.5 mg/ml soybean trypsin inhibitor) at 4 °C and minced using scissors. The tissue is homogenized (Polytron, setting no. 9 for two 15-s bursts), strained through gauze and centrifuged at 100 000 *g* for 15 min at 4 °C. The supernatant is discarded and the pellet resuspended in 20 vol of buffer and recentrifuged. The final pellet is diluted to a concentration of 1.25 mg/ml (original wet weight) in buffer and kept on ice until used. The incubation reaction is initiated by the addition of 100 µl of tissue to 96-well plates containing 150 µl of incubation buffer (50 mM Tris HCl, pH 7.4, and a final concentration of 5 mM $MgCl_2$, 5 mM dithiothreitol and 1% DMSO) with 60 pM final concentration of ^{125}I-BH-CCK_{8S}, and drug or vehicle. After a 30-min incubation the reaction is terminated by rapid filtration using a Skatron cell harvester (Skatron Instruments, Inc. VA) onto GF/B filters that were soaked for 2 h in 50 mM Tris HCl, 0.1 mg/ml bovine serum albumin. The filters are dried and counted on a Betaplate counter (Wallac Inc. Gaithersburg, MD) for 45 s per sample.

Povoski et al. (1994) reported cholecystokinin receptor characterization and cholecystokinin A receptor messenger RNA expression in transgenic mouse pancreatic carcinomas and dysplastic pancreas.

Yule et al. (1993), Blevins et al. (1994) recorded intracellular Ca^{2+} concentration signaling stimulated by cholecystokinin or by a partial CCK agonist in Chinese hamster ovary-CCK-A cells or in isolated pancreatic acini.

Ghanekar et al. (1997) established a Chinese hamster ovary cell line bearing the mouse type A cholecystokinin receptor.

CCK-A agonists were evaluated as anorectic agents (Pierson et al. 1997; Simmons et al. 1998).

CCK_B receptors occur predominantly in the central nervous system indicating involvement in behavioral functions (Moran et al. 1986; Wank et al. 1992b; Derrien et al. 1994; Schäfer et al. 1994) but also in pancreatic cancer cells (Smith et al. 1993; Zhou et al. 1992) and small lung cell cancer lines (Sethi et al. 1993).

For CCK_B receptor binding assays (Fossa et al. 1997), guinea pig cortex is homogenized with a Teflon homogenizer in 20 vol of 50 mM Tris HCl (pH 7.4) containing 5 mM $MgCl_2$ at 4 °C and centrifuged at 100 000 *g* for 30 min. The supernatant is discarded and the pellet resuspended and spun again. The pellet is diluted to a concentration of 10 mg/ml (original wet weight) with assay buffer (10 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, 130 mM NaCl, and 0.2 mg/ml bacitracin, pH 6.5) before use. The incubation reaction is initiated by the addition of 50 µl of tissue to 96-well plates containing 150 µl of assay buffer with 1% DMSO final concentration, 50 pM final concentration of ^{125}I-BH-CCK_{8S} and the appropriate concentration of drug or vehicle. Non-specific binding is estimat-ed using 1 µM CCK_{8S}. The reaction is terminated by spinning the plates using a H1 000B rotor at 3 000 rpm for 5 min at 4 °C. The pellet is washed with 200 µl of 50 mM Tris HCl and respun. The supernatant is again discarded, the pellet resuspended and the tissues harvested onto Betaplate filters soaked in 0.2% polyethylenimine for 2 h using a Skatron cell harvester (Skatron Instruments, Inc., VA). The filtermats are dried and counted on a Betaplate counter (Wallac Inc. Gaithersburg, MD) for 45 s per sample.

Lee et al. (1993) described cloning and characterization of the human brain cholecystokinin-B/gastrin receptor.

Kaufmann et al. (1993) studied the binding of a series of succinylated cholecystokinin tetrapeptide derivatives to different tissues and their effects on intracellular calcium mobilization ($[Ca^{2+}]_i$) in the human T-cell line Jurkat and rat pituitary (GH3) cells.

Durieux et al. (1989) described [^{3}H]pBC 264 as the first highly potent and very selective radioligand for CCK_B receptors.

Slaninova et al. (1995) recommended the radioiodinated CCK_8 analogue, SNF 8 702, as a selective radioligand for CCK_B receptors.

Knapp et al. (1990) found CCK-B receptor heterogeneity in various brain areas of the guinea pig using a highly selective CCK-B receptor radioligand.

Dunlop et al. (1996) described the functional characterization of a Chinese hamster ovary cell line trans-

fected with the human CCK-B receptor gene. Functional coupling in these cells was demonstrated using agonist stimulated mobilization of intracellular Ca^{2+}, measured with the FURA-2 technique.

CCK_A receptor antagonists have been described (Chang and Lotti 1986; Makovec et al. 1986; Evans 1993; Gully et al. 1993) as well as **CCK_B receptor antagonists** (Hill and Woodruff 1990; Ohtsuka et al. 1993; Pendley et al. 1993).

Selective non-peptide **CCK_B/gastrin receptor antagonists** have been described by Bertrand et al. (1994, Makovec et al. 1999; Takeuchi et al. 1999). Harper et al. (1999) analyzed some of them in radioligand binding assays in mouse and rat cerebral cortex.

Cholecystokinin dipeptoid antagonists with anxiolytic properties which bind preferably to CCK_B receptors have been reported by Boden et al. (1993).

CCK_A receptor antagonists (Ballaz et al. 1997) and **CCK_B receptor antagonists** (Revel et al. 1998) with anxiolytic-like activity in animal models were described.

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J.7.0.12 Acute experimental pancreatitis

PURPOSE AND RATIONALE

Acute interstitial pancreatitis can be induced in the rat by excessive doses of a pancreatic secretagogue (Lampel and Kern 1987) such as caerulein (Renner et al. 1986; Ito et al. 1991; Yazu et al. 1991).

PROCEDURE

Induction of pancreatitis

Male Wistar rats weighing 200 g are used. Pancreatitis is induced by caerulein given as 5 intraperitoneal injections of 40 µg/kg each at hourly intervals on day 1. Controls receive saline injections only. Treatment with potential drugs is started at day 1 and continued for 7 or 14 days. The rats are sacrificed after 1 or 2 weeks following caerulein injection.

Preparation of pancreatic acini

The rats are decapitated after overnight fasting and the pancreata are quickly removed. The pancreata are injected with digestion medium containing 170 U/ml collagenase and incubated for 15 min at 37 °C. The medium is changed for the second incubation for 60 min with 15 ml digestion medium containing 200 U/ml collagenase. Acini are dissociated by sequential passage through 4 pipettes of different diameters in a standard medium containing 0,1% soybean inhibitor. The suspension is filtered through a single layer of gauze and layered over 15 ml of 4% bovine albumin. After centrifugation for 2 min at 400 rpm, the pellet is washed 3 times with 20 ml of incubation medium containing 0.5 mM $CaCl_2$. Finally, the acini are resuspended in 10–20 ml of the incubation medium containing 10 mM HEPES, 145 mM NaCl, 4.7 mM KH_2PO_4, 1.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, 16.5 mM glucose, 0.1% bovine serum albumin, 0.01% soybean trypsin inhibitor, Eagles minimal essential amino acids and vitamins, adjusted to pH 7.4 and bubbled with 100% oxygen.

Measurement of [^{3}H]-thymidine uptake into pancreatic acini

Aliquots (5 ml) of the acini suspension are incubated at 37 °C for 30 min, and 6-[^{3}H]-thymidine (5 mCi/mmol) is added as a final concentration of 0.8 mCi/mol. After incubation for 60 min, 200 ml aliquots are filtered by vacuum through Whatman GF/B glass fiber filters. Filters which trapped the acini are washed with 15 ml ice-cold buffer. After adding 5 ml of Aquazol II to each vial, the radioactivity of the filters is counted with a scintillation spectrometer.

Measurement of amylase secretion from pancreatic acini

Acini are resuspended in 5 ml of the incubation medium and pre-incubated at 37 °C for 30 min. Carbachol at concentrations between 10^{-7} and 10^{-4} M or CCK-8 at concentrations between 10^{-11} and 10^{-8} M is added and the acini suspension is incubated at 37 °C for an additional 30 min. Triplicate 300 ml aliquots are centrifuged for 5 s at 3 000 rpm. The amylase activity in the supernatant is measured by a commercial amylase test. Triplicate 500 ml aliquots are sampled before adding the secretagogue and the amylase contents (total amylase) are measured after sonification.

EVALUATION

Thymidine uptake is compared between control, caerulein treated animals and animals treated with drugs. Dose-response curves of amylase secretion after carbachol and CCK-8 are established for each group. Moreover, amylase concentrations in the acini are compared.

MODIFICATIONS OF THE METHOD

Griesbacher and Lembeck (1992), Griesbacher et al. (1993) studied the prevention of **caerulein-induced** experimental acute pancreatitis in the rat by the bradykinin antagonist HOE 140.

Several authors (Ito et al. 1994; Ogden et al. 1994; Liu et al. 1995; Sledzinski et al. 1995; Weidenbach et al. 1995; Chen et al. 1996; Lembeck and Griesbacher 1996; Asano et al. 1997; Ito et al. 1997) used different dose regimens of caerulein administration to induce acute pancreatitis in rats.

Huch et al. (1995) induced necrotizing pancreatitis by intraductal infusion of low-dose glycodeoxycholic acid (10 mmol/l) followed by intravenous cerulein (6 µg/kg/h) for 6 h.

Several other chemicals and drugs may induced acute pancreatitis (Vogel 1994).

Merkord et al. (1997) studied the pathogenesis and the time course of lesions of acute interstitial pancreatitis in rats induced by intravenous injection of 6 mg/kg **dibutyltin dichloride** (DBTC), an organotin compound used in chemical industry and in veterinary medicine. First, the cytotoxic effects on the biliopancreatic duct epithelium lead to epithelial necrosis with obstruction of the duct, subsequent cholestasis, and interstitial pancreatitis; and second, the hematogenic effects of DBTC cause direct injury of pancreatic cells followed by interstitial edema and inflammation. A chronic course is found when the obstruction of the duct and cholestasis persist.

Destruction of acinar cells has been found after the administration of **ethionine** in rats (Herman and Fitzgerald 1962).

Niederau et al. (1985), Neuschwander-Tetri et al. (1994), Norman et al. (1995), Van Laethem et al. (1995), Taniguchi et al. (1996) described acute necrotizing pancreatitis induced by caerulein in **mice**.

Pancreatitis can be induced in mice by a **choline-deficient diet** (Lombardi et al. 1975; Niederau et al. 1990). Mice weighing 10–14 g are fed regular laboratory chow ad libitum before the experiment. A choline-deficient diet supplemented with 0.5% ethionine is given for a period of 66 h, after which it is replaced by regular chow.

Lake-Bakaar and Lyubsky (1995), Hirano (1997), Niederau et al. (1995) induced acute pancreatitis in female Swiss-Webster mice by feeding a choline- and methionine-deficient diet supplemented with 1% ethionine.

Emanuelli et al. (1994) demonstrated that a single injection of endotoxin (lipopolysaccharides, *E. coli* 0111-B4) into the superior pancreaticoduodenal artery of **rabbits** induced a dose-dependent acute necrotizing pancreatitis.

Watanabe et al. (1993) induced acute hemorrhagic pancreatitis in rats by surgically **closing a 1 cm length of duodenal loop** at points proximal and distal to the orifice of the pancreatic duct for 6 h with bypassing the bile from the liver hilus distal to the closed loop. The effects of Hoe 140, a bradykinin antagonist, were studied.

Ha et al. (1994, 1996) used the closed duodenal loop technique to study the role of endogenous and exogenous cholecystokinin in experimental pancreatitis and the effect of a cholecystokinin receptor antagonist on the early stage of the healing process in acute pancreatitis.

Kimura et al. (1988) found beneficial effects of a cholecystokinin A receptor antagonist in three methods of acute experimental pancreatitis: pancreatitis in mice induced by 6 intraperitoneal injections of 50 µg/kg caerulein, necrotizing pancreatitis in rats induced by injection of sodium taurocholate into the common bile duct followed by 4 subcutaneous injections of 50 µg/kg caerulein, and in closed duodenal loop induced pancreatitis in rats.

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J.7.0.13 Taurocholate-induced pancreatitis in the rat

PURPOSE AND RATIONALE

Retrograde infusion of bile salts into the pancreatic duct induces severe necrotizing pancreatitis indicated by reduced amylase output and histological changes.

PROCEDURE

Male Sprague-Dawley or Wistar rats weighing 200–300 g are anesthetized by i.m injection of a mixture of 87 mg/kg ketamine and 13 mg/kg xylazine. After laparatomy, the pancreaticobiliary duct is cannulated through the duodenal papilla with a polyethylene catheter (P10, Clay Adams) which is introduced by means of a puncture in the duodenum. A precision pump is used to infuse 0.6 ml 5% sodium taurocholate into the pancreaticobiliary duct during a 10-min period at an infusion rate of 6 ml/h. The catheter is then withdrawn and the abdominal cavity surgically closed.

After various time intervals (several hours up to 2 weeks), the animals are anesthetized and the pancreaticobiliary duct is cannulated again. The response to various doses of caerulein is measured.

EVALUATION

The degree of amylase output (mg/h) is taken as parameter. Dose response curves after various doses of caerulein are established.

MODIFICATIONS OF THE METHOD

The effects of various agents on experimental pancreatitis induced by retrograde intraductal injection of **taurocholate** solution or bile acid in rats have been studied by various authors (Bielecki et al. (1994), Hietaranta et al. 1995; Nakae et al. 1995; Niederau et al. 1995; Kimura et al. 1996; Mithofer et al. 1996; Paran et al. 1996; Tachibana et al. 1996; Norman et al. 1997; Plusczyk et al. 1997; Manso et al. 1998).

Tanaka et al. (1995), Sakai (1996) induced necrotizing pancreatitis by retrograde injection of **deoxycholate** solution into the biliopancreatic duct of rats.

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J.7.0.14 Chronic pancreatitis

PURPOSE AND RATIONALE

Chronic pancreatitis was induced by various means in rats (Zhou 1990, 1994; Goto et al. 1995; Puig-Diví et al. 1996), golden hamsters (Rutishauser et al1991, 1995), cats (Reber et al. 1992, 1999; Zhao et al. 1996), dogs (Hayakawa et al. 1993; Tanaka et al. 1994, 1998), and pigs (Vinter-Jensen et al. 1997).

Puig-Diví et al. (1996) induced chronic pancreatitis in rats by trinitrobenzene sulfonic acid infusion into the pancreatic ducts.

PROCEDURE

Male Sprague Dawley rats weighing 300–350 g are anesthetized with ketamine (100 mg/kg i.p.) after an overnight fast. Access to the pancreas is gained through a ventral midline incision. The duodenum is opened and the biliopancreatic duct cannulated through the papilla using polyethylene tubing (PE 10). The biliopancreatic secretion is allowed to drain freely for 15 min. To prevent liver damage, the duct is tied close to the liver. Retrograde infusion is initiated by means of a controlled pressure device that uses the height of a liquid column (trinitrobenzene sulfonic acid or vehicle) in a vertical pipette connected to the infusion cannula to control the intraductal pressure and infusion volume. Ductal pressure is never allowed to exceed 20 cm H_2O. In the treated group 0.4 ml of 2% trinitro-benzene sulfonic acid in phosphate buffered saline + 10% ethanol (pH 8) is infused. Ethanol is employed as an epithelial "barrier breaker" to facilitate trinitrobenzene sulfonic acid penetration into the tissue. Rats in the control group undergo the same procedure, except that trinitrobenzene sulfonic acid in absent from the infusion medium.

The total exposure time of the gland to the instillate is 60 min, followed by a washout period of 30 min. Ligatures are then released, the duodenum and the abdominal wall are sutured, and the animals are kept under observation for 2 h after surgery. Rats are then transferred to individual cages, where they are fasted for 24 h. Thereafter they receive standard chow and their weight gain is recorded weekly.

Blood is withdrawn after various time intervals by cardiac puncture under light ether anesthesia for determination of glucose and serum α-amylase (EPS test, Boehringer Mannheim GmbH).

Groups of treated rats and controls are sacrificed at 3, 4, and 6 weeks after the surgical procedure. Pancreata are fixed in 10% neutral buffered formaldehyde and embedded in paraffin. Several sections are cut and stained with haematoxylin-eosin for light microscopy evaluation. The degree of periductal and intralobular fibrosis, patchy acute and chronic inflammatory cell infiltrates, common duct stenosis, and segmentary gland atrophy is evaluated.

EVALUATION

Data are expressed as means ±SEM. A two-tailed Student's *t*-test for unpaired values is used for statistical comparison of mean values of serum amylase, glucose and rat weight.

MODIFICATIONS OF THE METHOD

Chung and Richter (1971), Zhou et al. (1990, 1994) induced chronic pancreatitis in rats by **ligation of the pancreatic duct**.

Injection of oleic acid (Goke et al. 1989; Goldstein et al. 1989; Andersen et al. 1994; Kakugawa 1996; Seymour et al. 1995, 1998) or of a viscous solution of zein – oleic acid – linoleic acid (Kataoka et al. 1998) into the pancreatic duct was used to induce chronic pancreatitis in rats.

Goto et al. (1995) described a chronic pancreatitis model with diabetes induced by intraperitoneal **cerulein** injection plus water immersion stress in rats.

Pancreatic blood flow was measured in **cats** with chronic pancreatitis induced by partial ligation of the pancreatic duct by Austin et al. (1980), Reber et al. (1992, 1999), Widdison et al. (1992).

Zhao et al. (1996, 1998) induced progressing lesions of chronic pancreatitis in cats by intraductal injection of alcohol or by a combination of intraductal and intraparenchymal injection of ethanol together with partial obstruction of the main pancreatic duct to 70% of its original lumen by fixation of a small catheter in the papilla.

Hayakawa et al. (1993) induced pancreatolithiasis in **dogs** by partial obstruction of the major pancreatic duct.

Rats of the diabetic strain WBN/Kob (Tsuchitani et al. 1985; Nakama et al. 1985) develop **spontaneously chronic pancreatitis** (Ohashi et al. 1990; Sato et al. 1993; Sugiyama et al. 1996a,b; Arai et al. 1998; Ito et al. 1998) with pancreatic fibrosis and parenchymal destruction and both endocrine and exocrine pancreatic dysfunction (see also K.2.0.2).

Transgenic mice overexpressing $TGF_{\beta 1}$ (Sanvito et al. 1995) develop tissue changes in the pancreas resembling changes found in chronic pancreatitis.

Shetzline et al. (1998) identified target tissues of pancreatic polypeptide using an *in vivo* radioreceptor assay in order to further elucidate the function of this hormone.

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J.8 Liver function

J.8.1 Hepatocellular function

J.8.1.1 Hepatitis in Long Evans Cinnamon rats

PURPOSE AND RATIONALE

The Long Evans Cinnamon strain of rats has been recommended as a useful model to study genetically transmitted fulminant hepatitis and chronic liver disease (Yoshida et al. 1987; Hawkins et al. 1995). The underlying cause is thought to be due to excessive copper accumulation in the liver of Long Evans Cinnamon rats, thus making this animal a model for Wilson's disease in humans (Okayasu et al. 1992). Chelation therapy or feeding a copper-deficient diet can ameliorate the symptoms in Long Evans Cinnamon rats and Wilson's disease (Togashi et al. 1992).

PROCEDURE

Male Long Evans Cinnamon rats obtained from a commercial breeder at an age of 5 weeks are housed in temperature- und humidity-controlled rooms at a 12 : 12 light/dark cycle. Groups of 6–10 rats are given different diets based on a 15% purified egg protein diet and supplemented with vitamins or drugs. Drugs are applied via minipumps intraperitoneally implanted under ether anesthesia.

The occurrence of jaundice is easily observable as the time when the ears and tail turn yellow and the urine becomes bright orange, staining the fur in the lower abdominal region. Usually, the jaundice progressively worsens, ending in death of the animal within about a week. Incidence of jaundice and mortality vs. time are used as parameters.

EVALUATION

Statistics are performed using StatView II software package for Student's *t*-test, ANOVA, and the Scheffé F-test for comparison between means. All data are expressed as means. A p-value < 0.05 is used as the threshold of significance.

MODIFICATIONS OF THE METHOD

Several drugs which are known to be effective or which are potentially effective in treatment of Wilson's disease were studied in this animal model, such as D-penicillamine (Togashi et al. 1992; Yokoi et al. 1994; Shimizu et al. 1997), trientine (= triethylenetetramine) (Iseki et al. 1992; Sone et al. 1996; Yamamoto et al. 1997), tetrathiomolybdate (Ogra et al. 1995; Suzuki 1997; Sugawara et al. 1999), or the investigative drug TJN-101 (Yokoi et al. 1995).

The interferon-γ transgenic mouse which carries the mouse INF-γ gene develops chronic hepatitis from the age of 6–10 weeks and was recommended by Okamoto et al. (1999) as a model for **chronic hepatitis**.

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J.8.1.2 Temporary hepatic ischemia

PURPOSE AND RATIONALE

Hepatocellular function is altered by temporary hepatic ischemia as occurring during surgical management of acute hepatic trauma and being essential during hepatic transplantation. To study this, total hepatic ischemia in rats is produced by placing a ligature around the hepatic artery, portal vein, and the common bile duct.

PROCEDURE

Hepatic ischemia procedure

Male albino Holtzman rats, weighing 300–350 g are fasted for 16 h prior to the experiment but are allowed water ad libitum. The rats are anesthetized lightly with ether and the abdominal cavity is opened through a midline incision. Splenectomy is performed following which a temporary extracorporal splenofemoral shunt is established between the splenic vein and the right femoral vein using a PE-190 tubing. To insure total hepatic ischemia, the portal vein as the hepatic artery and the bile duct are occluded by placing a tourniquet around the vessels. Blood pressure is measured via a catheter inserted into the right femoral artery. After heparinization (10 mg/kg), hepatic ischemia is produced for 60 min. During the ischemic period, 0.7 ml of saline is given i.v. at 20-min intervals for volume replacement. At the end of the 60-min ischemic period, the tourniquet around the portal vein, hepatic artery and the bile duct is removed in order to reestablish blood flow to the liver. The abdominal incision is then closed and the animals receive either saline (nontreated) or the drug. Following administration of saline or the drug the catheters are removed and the animals are returned to their home cages.

Sham-operated animals are prepared exactly in the same manner except that the tourniquet around the portal vein, hepatic artery, and the bile duct are not placed.

Measurement of indocyanine green clearance

Three hours following the end of ischemia, the experimental as well as the sham-operated rats are lightly anesthetized with ether and a femoral artery and vein of each animal cannulated. Sodium heparin (400 units) is given i.v. and the animals are allowed to awake. Indocyananine green is given i.v. at 5 mg/kg (low) or 25 mg/kg (high) to the animals via the femoral vein and 0.2 ml arterial blood samples are taken at 5, 6, 8, 10, 12, 15, 18, and 20 min later. The blood samples are diluted with 0.8 ml of 1% bovine serum albumin in normal saline and centrifuged at 6 000 rpm for 20 min at 4 °C. The spectrophotometric absorbance of the supernatant is read at 800 nm and the indocyanine green concentration determined from a standard curve.

EVALUATION

The $t_{½}$ of indocyanine green clearance is computed for each animal using a computer program which calculates the least square line of log indocyanine green vs. time. Mean and standard errors for each group are compared using the Student *t*-test.

MODIFICATIONS OF THE METHOD

Daemen et al. (1989) compared the electromagnetic versus the microsphere and the clearance method for liver blood flow measurement in the rat.

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J.8.1.3 Model for direct transhepatic studies in dogs

PURPOSE AND RATIONALE

A chronic conscious dog model for repeated transhepatic studies over a period of 6–8 weeks was developed by O'Brien et al. (1991). This model can be applied to the study of the hepatic effects of pancreatic hormone secretion and glucose metabolism, to studies of the hepatic mechanisms associated with high first-pass metabolism and food interactions of drugs (Semple et al. 1990), and to studies of insulin balance in dogs that have undergone previous pancreatectomy and islet cell auto-transplantation.

PROCEDURE

Four silastic catheters, 0.062 in. ID × 0.125 in. OD (lengths: carotid 70 cm; jugular 70 cm; hepatic 80 cm; and portal 70 cm) and two ultrasonic transit time flow probes suitable for long-term implants (Burton and Gorewit 1984), 4 mm for the hepatic artery and 6 mm for the portal vein, are cleaned with chlorhexidine scrub and rinsed with distilled water. Double velour dacron cuffs are placed 15 cm from the external ends of all the devices.

Male dogs weighing 20–25 kg are sedated and anesthetized with a 2% halothane/L O_2 mixture. Skin interface sites and subcutaneous pockets for placement of catheters are prepared. After skin closure the external ends of the catheters are sealed. Then the catheters and flow probes are placed into the abdomen by retrieving them from the subcutaneous pockets. First, the hepatic artery flow probe is placed, then, the portal venous flow probe inserted. To eliminate extrahepatic blood flow, the gastroduodenal artery is ligated. Then, the portal vein and the hepatic vein catheters are placed. After ensuring catheter patency, the abdomen is closed. Finally, a carotid artery catheter and a jugular venous catheter are placed.

EVALUATION

Blood samples can be withdrawn from the catheters placed into the carotid artery, the right external jugular vein, the portal vein, and the hepatic vein. Blood flow is measured by flow probes in the hepatic artery and the hepatic portal vein.

The following values are measured:

- Plasma flow in the portal vein and plasma flow in the hepatic artery,
- drug concentration in the portal vein, in the hepatic artery, in the hepatic vein, and in the right external jugular vein.

From these data plasma flux in the portal vein, in the hepatic artery, and in the hepatic vein and the interval areas under the curve for these vessels are calculated.

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J.8.2 Liver cirrhosis and necrosis

J.8.2.1 General considerations

Various factors induce liver cirrhosis in man, such as alcoholism, viral hepatitis, intoxications, bile duct dis-

orders, inborn diseases, and others. In the process leading to cirrhosis, accumulation of connective tissue and parenchymal regeneration are competing events. Excessive formation of connective tissue with collagen overproduction in the liver reduces hepatic blood flow, impairs the metabolic functions of the liver, and increases portal vein pressure. These mechanisms result in hepatic failure, esophageal bleeding, portal hypertension and ascites. Therefore, the search for agents to prevent liver cirrhosis is focused on inhibitors of excessive connective tissue formation in the liver (Kervar et al. 1976; Nolan et al. 1978). The main component of connective tissue formed as a response to chronic injury is collagen. The collagenous fibers consist of triple helical molecules. Their formation depends on the presence of hydrogen bonds involving the post-translationally hydroxylated amino acid hydroxyproline. If the number of hydrogen bonds is reduced due to a decrease of hydroxylated amino acids, the resulting collagen can not form the triple helix and is degraded instead of being deposited in the extracellular matrix.

Insoluble collagen is responsible for most of the mechanical functions of connective tissue, e.g., bone, tendon and skin, being influenced by hormones, desmotropic drugs, such as D-penicillamine, and by maturation and age (Vogel 1969, 1972, 1974a,b, 1976, 1978, 1980, 1989; Bickel et al. 1990, 1991). The aim of fibrosuppressive compounds is to reduce only the excessive formation of insoluble collagen in the liver leaving collagen synthesis and turnover in other tissues intact. Fibrosuppressive effects by inhibition of proline hydroxylation can be screened with *in vitro* methods, however, the desired organ specificity has to be tested in models of liver cirrhosis and fibrosis *in vivo* together with functional studies of the connective and supporting tissue. Detailed description of methods for studying collagen metabolism and mechanical function of connective tissue is given in the Sect. M.2.1.3 (hormones).

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J.8.2.2 Inhibition of proline hydroxylation

PURPOSE AND RATIONALE

The thermal stability of the triple helix of collagenous proteins is crucially dependent upon the intramolecular hydrogen bonds involving the 4-hydroxyproline residues synthesized by the enzyme prolyl 4-hydroxylase. This makes the enzyme a possible target for therapeutic antifibrotic agents.

PROCEDURE

Enzyme activity is assayed in sealed test tubes. The reaction volume of 1 ml contains 50 mM Tris buffer, pH 7.5, 10–100 mM (60 000 d.p.m.) 2-oxo[1-^{14}C]glutarate, 1–50 mM $FeSO_4$, 0.1–1 mM ascorbate, 10–100 mg $(Pro\text{-}Pro\text{-}Gly)_{10}$, 0.1 mg catalase, 2 mg bovine serum albumin, 100 mM dithiotreitol, 0.05–0.2 mg enzyme, and inhibitors in various concentrations. After incubation at 37 °C for 30 min, the generated $^{14}CO_2$ is trapped and determined.

EVALUATION

Inhibition modes are determined by plotting 1/v versus 1/concentration of the variable substrate (Lineweaver-Burk plot). The K_i values are derived from a secondary transformation (slopes or intercepts vs. inhibitor concentrations). The lines of best fit for primary plots and secondary transformations are calculated by using the method of least squares. The mean K_i value of 4–6 experiments is calculated.

MODIFICATIONS OF THE METHOD

The collagen hydroxylases lysyl hydroxylase and prolyl 3-hydroxylase have similar reaction mechanisms as prolyl 4-hydroxylase, differing only in the specificity for the amino acid sequence of the substrate (Kivirikko and Myllylä 1982). Instead of $(Pro\text{-}Pro\text{-}Gly)_{10}$, 50–500 mg Arg-Ala-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly are used.

The activity of prolyl 3-hydroxylase is assayed in a reaction volume of 1.5 ml containing 50 mM Tris buffer, pH 7.5, 1 000 000 d.p.m. of biologically obtained [2,3-^{3}H]proline-labeled protocollagen substrate in which all proline residues recognized by proline 4-hydroxylase are converted to hydroxyproline, 2 mM ascorbate, 0.2 mg/ml catalase, 2 mg/ml bovine serum albumin, 15 mM 2-oxoglutarate, 50 mM Fe^{2+}, 100 mM dithiothreitol, 0.2–2 mg enzyme, and inhibitors at various concentrations. After incubation at 37 °C for 30 min, the reaction is stopped by addition of 0.5 ml 10% trichloroacetic acid (w/v). The reaction mixture is then distilled, and 1.6 ml of ^3H_2O is counted for radioactivity.

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J.8.2.3 Influence on collagen synthesis in human skin fibroblasts

PURPOSE AND RATIONALE

Secretion of collagen by fibroblasts and other cells capable of synthesizing extracellular matrix is dependent on the hydroxylation of proline residues by prolyl 4-hydroxylase. This enzyme is located in the cisternae of the endoplasmic reticulum. An agent aimed at inhibition of this enzyme must therefore pass both the external cell membrane and the endoplasmic reticular membrane. Organ specificity of a prolyl 4-hydroxylase inhibitor can be achieved by applying a prodrug which can be converted to the active agent only in cells of specialized tissues, e.g., in the liver, but not generally in fibroblasts.

PROCEDURE

Confluent cultures of human skin fibroblasts are preincubated for 24 h at 37 °C without serum in glutamine-free Dulbecco's minimal essential medium supplemented with 50 mg/ml sodium ascorbate, 60 mg/ml 3-aminopropionitrile, and 100 U/ml penicillin G. The cells are then exposed to the potential inhibitor at various concentrations for 20 min, followed by the addition of 2 mCi [U-^{14}C]proline/ml. The incubation is continued for 5 h at 37 °C. Then, the cells are separated from the medium. After removal of non-incorporated [^{14}C]proline, the proteins from medium and cells are hydrolyzed, and the hydroxyproline content is determined by amino acid analysis. The total incorporation of radioactivity serves as marker for protein synthesis.

EVALUATION

Two individual samples are taken for each concentration of the inhibitor and 6 samples for the controls. Proline incorporation is expressed as % of control radioactivity. Hydroxyproline synthesis is expressed as relative Hyp/Pro ratio according to the formula:

$$(Hyp/Pro_{sample} / Hyp/Pro_{control}) \times 100$$

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J.8.2.4 Influence on collagen synthesis in chicken calvaria

PURPOSE AND RATIONALE

To find fibrosuppressive agents for therapeutic use, it is necessary to have prodrugs which cross cell membranes readily and are converted intracellularly to the active agent. Proinhibitors which are cleaved hydrolytically can suppress collagen synthesis in chicken calvaria.

PROCEDURE

Calvaria are removed from chicken embryos, age 15 days, and washed for 3 min with DMEM at 37 °C. They are then transferred into Pyrex tubes (8–10 calvaria/tube) containing 3 ml medium supplemented with 2 mM glutamine, 6 mCi [U-^{14}C-]proline, and various concentrations of the inhibitor. The samples are incubated for 1.5 to 6 h at 37 °C. The experiment is terminated by placing the tubes in ice and separation of calvaria from the culture medium. The calvaria are washed once with 3 ml of fresh medium, which is then pooled with the incubation medium. Bovine serum albumin and phenyl methyl sulfonyl fluoride are added (final concentration 1 mg/ml and 6 mg/ml, respectively). The calvaria are extracted for 16 h with 25 ml of 0.5 M acetic acid.

The following procedure is identical for the medium and the calvaria extracts. The samples are extensively dialyzed against 0.5 M acetic acid at 4 °C. Aliquots are withdrawn for SDS-PAGE1 and the triple-helix stability assay is performed. The remaining material is lyophilized, resuspended in 2 ml 6 N HCl and hydrolyzed at 105 °C for 24 h. After evaporation of the acid, the samples are dissolved in 2 ml H_2O, and the hydroxyproline content is determined according to Juva and Prockop (1967).

In order to study the degree of collagen hydroxylation and proportion of collagen biosynthesis, calvaria are incubated in the presence of 10 mCi [3,4-^{3}H]-proline/ml and 2 mCi [U-^{14}C]-proline/ml for 3 h under the conditions described above. After lyophilization, aliquots of media and calvaria samples are digested with collagenase according to Peterkofsky et al. (1982). The degree of hydroxylation is calculated from the ^{3}H/^{14}C ratio in the digested material; the amount of collagen as a proportion of total protein synthesis is determined by the relation of collagenase degradable vs. collagenase resistant radioactivity. The stability of the extracted collagenous material against digestion by a trypsin/chymotrypsin mixture is tested according to the procedure proposed by Bruckner and Prockop (1981): An aliquot of either culture medium or calvaria extract is incubated for 15 min at a temperature between 25 °C and 45 °C in a total volume of 800 µl of 0.04 M NaCl/0.1 Tris, pH 7.4. After quenching to 0 °C the sample is digested with 200 µl of a mixture of each 1 mg trypsin and chymotrypsin/ml buffer for 15 min at room temperature. One hundred µl of 1 mg BSA/ml buffer is added, and the protease-resistant radioactivity, consisting of triple-helical collagen, is precipitated with 100 µl 100% trichloroacetic acid (w/v). The sample is transferred in total to a Schleicher and Schüll OE 67 filter paper of 2.5 cm diameter. The digested material is removed by repeated washing with cold 5% trichloroacetic acid and methanol. The filters are then dried and the radioactivity is determined. Unhydrolyzed samples are studied by SDS-polyacrylamide gel electrophoresis, and autofluorography. The morphologic appearance of the control and treated cultures is studied by electron microscopy.

EVALUATION

IC_{50}-values of hydroxyproline synthesis are read graphically from concentration response curves. Total protein synthesis is estimated as the incorporation of proline; the mean ±standard deviation is calculated from four samples.

MODIFICATIONS OF THE METHOD

Canalis et al. (1977) used cultured calvaria from 21 day fetal rats to study the effects of insulin and glucagon on bone collagen synthesis.

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J.8.2.5 Allyl alcohol induced liver necrosis in rats

PURPOSE AND RATIONALE

Administration of allyl alcohol induces focal liver necrosis in rats which can be partially prevented by treatment with several drugs such as antibiotics.

PROCEDURE

Female Wistar rats weighing 120–150 g are used. At 8:00 A.M. of the first day food but not water is withdrawn. At 3:00 P.M. the compounds to be tested for protective activity are administered i.p. or orally. One hour later, the animals are dosed orally with 0.4 ml/kg of a 1.25% solution of allyl alcohol in water. At 8:00 A.M. of the second day the treatment with the potentially protective drugs is repeated. Food but not water is withheld until the third day. At 8:00 A.M. of the third day the animals are sacrificed and the liver removed. The parietal sides of the liver (left, medium and right lobe and lobus caudatus) are checked using a stereomicroscope with 25 times magnification. Focal necrosis is observed as white-green or yellowish hemorrhagic areas clearly separated from unaffected tissue. The diameter of the necrotic areas is determined using a ocular-micrometer. These values are added for each animal to obtain an index for necrosis.

EVALUATION

Using 10 animals for controls and for each treatment group, the mean of necrosis index is calculated and compared with Student's *t*-test. The protective effect is expressed as percentage decrease of the necrosis index versus controls.

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J.8.2.6 Carbontetrachloride induced liver fibrosis in rats

PURPOSE AND RATIONALE

Chronic administration of tetrachloride to rats induces severe disturbances of hepatic function together with histologically observable liver fibrosis.

PROCEDURE

Groups of 20 female Wistar rats with a starting body weight of 100–150 g are used. The animals are treated orally twice a week with 1 mg/kg carbontetrachloride, dissolved in olive oil 1 : 1, over a period of 8 weeks. The animals are kept under standard conditions (day/night rhythm 8:00 A.M. to 8:00 P.M., 22 °C room temperature, standard diet, e.g. Altromin® 1 321 pellets, and water ad libitum). Twenty animals serve as controls receiving olive oil only, 40–60 animals receive the carbontetrachloride only, and groups of 20 rats receive in addition the compound under investigation in various doses by gavage twice daily (with the exception of the weekends, when only one dose is given) on the basis of the actual body weight. The animals are weighed weekly.

At the end of the experiment (8 weeks), the animals are anesthetized and exsanguinated through the caval vein.

In the *serum*, the following parameters are determined:

- Total bilirubin,
- total bile acids,
- 7S fragment of type IV collagen,
- procollagen III N-peptide.

The following organs are prepared for determination of hydroxyproline:

- Liver,
- kidney,
- aortic wall, and
- tail tendons.

The specimens of the organs are weighed and completely hydrolyzed in 6 N HCl. Hydroxyproline is measured by HPLC and expressed as mg/mg wet weight of the organs.

To measure **mechanical properties of connective tissue**, the following organs are prepared: Femur and tibia of both sides, tail tendons, and strips from dorsal skin (Bickel et al. 1990, 1991; detailed description of the methods see N.2.1.3). Furthermore, the influence on the healing process of skin wounds is studied (Method see N.2.1.3.5).

For **histological analysis**, 3–5 pieces of the liver weighing about 1 g are fixed in formalin and Carnoy solution. Three – 5 sections of each liver are embedded, cut and stained with azocarmine aniline blue (AZAN) and evaluated for the development of fibrosis using a score of 0–IV.

Grade 0:	Normal liver histology.
Grade I:	Tiny and short septa of connective tissue without influence on the structure of the hepatic lobules.
Grade II:	Large septa of connective tissue, flowing together and penetrating into the parenchyma. Tendency to develop nodules.
Grade III:	Nodular transformation of the liver architecture with loss of the structure of the hepatic lobules.
Grade IV:	Excessive formation and deposition of connective tissue with subdivision of the regenerating lobules and with development of scars.

EVALUATION

For detection of significant differences ($p < 0.05$), the unpaired *t*-test is used. For comparison of the scores in the histological evaluation, the χ^2-test is used.

MODIFICATIONS OF THE TEST

Instead of chronic intoxication with carbontetrachloride resulting in liver fibrosis, acute hepatocellular damage can be achieved by short term application of carbontetrachloride.

Wistar rats with a starting body weight of 120–150 g are treated daily for 5 days with various oral doses of the compound under investigation. From day 2 to day 5 (4 applications) the rats receive by gavage a dose of 1 mg/kg carbontetrachloride dissolved in olive oil (1 : 1). Blood is withdrawn every day and the aminotransferases ALAT and ASAT, as well as total bilirubin are determined in the serum.

Kawaura et al. (1993) produced liver cirrhosis with ascites in **dogs** by administration of 2 ml carbontetrachloride per kg body weight once a week for 4 weeks. Eight weeks afterwards, the supradiaphragmatic inferior vena cava was constricted to 50% resulting in ascites formation of 500 to 1 000 ml. The dogs could be treated by ligation of the common hepatic artery and hepatocyte inoculation into the spleen.

Wirth et al. (1997) studied the effects of a bradykinin B_1 receptor antagonist in rats with CCl_4-induced liver cirrhosis.

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J.8.2.7 Bile duct ligation induced liver fibrosis in rats

PURPOSE AND RATIONALE

Bile duct ligation in rats induces liver fibrosis which can be evaluated by histological means and by determination of serum collagen parameters.

PROCEDURE

Male Sprague Dawley rats weighing approximately 250 g are anesthetized with ketanest (Rompun®). Laparatomy is performed under antiseptic conditions. A mid-line incision in the abdomen is made from the xiphosternum to the pubis, exposing the muscle layers and the linea alba, which is then incised over a length corresponding to the skin incision. The edge of the liver is then raised and the duodenum pulled down to expose the common bile duct, which pursues an almost straight course of about 3 cm from the hilum of the liver to its opening into the duodenum. There is no gallbladder, and the duct is embedded for the greater part of its length in the pancreas, which opens into it by numerous small ducts. A blunt aneurysm needle is passed under the part of the duct selected, stripping the pancreas away with care, and the duct is divided between double ligatures of cotton thread. The peritoneum and the muscle layers as well as the skin wound are closed with cotton stitches.

The animals receive normal diet and water ad libitum throughout the experiment. Groups of 5–10 animals receive the test compound in various doses or the vehicle twice daily for 6 weeks. Then, they are sacrificed and blood is harvested for determination of bile acids, 7S fragment of type IV collagen, and procollagen III N-peptide. The liver is used for histological studies and for hydroxyproline determinations. Control animals show excessive bile duct proliferation as well as formation of fibrous septa. The picture is consistent with complete biliary cirrhosis.

EVALUATION

For detection of significant differences ($p < 0.05$), the unpaired *t*-test is used.

MODIFICATIONS OF THE METHOD

Alpini et al. (1994) found an upregulation of secretin receptor gene expression in rat cholangiocytes after bile duct ligation.

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J.8.2.8 Galactosamine induced liver necrosis

PURPOSE AND RATIONALE

Single dose or a few repeated doses of D-galactosamine cause acute hepatic necrosis in rats (Decker and Keppler 1972). Prolonged administration leads to cirrhosis (Lesch et al. 1970).

PROCEDURE

For induction of acute experimental hepatotoxicity, divided doses of 100 to 400 mg/kg D-galactosamine are injected to rats i.p. or i.v. during one day.

For induction of liver cirrhosis, male Wistar rats weighing 110–180 g are injected intraperitoneally three times weekly with 500 mg/kg D-galactosamine over a period of one to 3 months. Potential protective substances are administered orally with the food or by gavage every day. The rats are sacrificed at various time intervals and the livers obtained by autopsy.

EVALUATION

The livers are evaluated by light microscopy and immunohistology using antibodies against macrophages, lymphocytes and the extracellular matrix components, e.g., laminin, fibronectin, desmin, collagen type I, III, and IV. The extent of liver cell necrosis and immunoreactivity for macrophages, lymphocytes and the extracellular matrix components is graded semiquantitatively on a 0 to 4+ scale (0 = absent, 1+ = trace, 2+ = weak, 3+ = moderate, and 4+ = strong). Furthermore, serum enzyme activities, such as GOT and GPT, are determined.

MODIFICATIONS OF THE METHOD

Other agents used to induce experimental cirrhosis are ethionine, thioacetamide (Dashti et al. 1996), dialkylnitrosamines, tannic acid, aflatoxins, pyrrolidizine alkaloids, and hepatotoxic components from mushrooms, such as amatoxins and phallotoxins (Zimmerman 1976).

Bruck et al. (1996) found an inhibition of thioacetamide-induced liver cirrhosis in rats by a nonpeptidic mimetic of the extracellular matrix-associated Arg-Gly-Asp epitope.

Intrahepatic cholestasis can be induced by alpha-naphthylisothiocyanate in rats (Krell et al. 1982).

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J.9 Eviscerated animals

J.9.1 Evisceration in rats

PURPOSE AND RATIONALE

Eviscerated animals have been used to study the influence of hormones and other drugs on carbohydrate disposal (Russell 1942; Creutzfeldt et al. 1961; Wick and Drury 1963; Willms et al. 1969; Tanira and Furman 1999).

"Functional" evisceration has been performed by most authors (Russell 1942; Creutzfeldt et al. 1961; Creutzfeldt and Deuticke 1962; Willms et al. 1969; Penhos et al. 1970; Smith 1986; Smith et al. 1990; Tanira and Furman 1999). The abdominal viscera, with the exception of the liver, are removed, and the blood vessels supplying the liver – the coeliac axis and the portal vein – are tied and cut.

PROCEDURE

Male Wistar rats weighing 180 to 280 g are allowed free access to food and water until the experiment. They are anesthetized with 55 mg/kg pentobarbitone sodium i.p. Cannulae are placed in the left femoral vein and in the abdominal aorta via the left femoral artery.

The abdominal cavity is opened and the anterior mesenteric artery, posterior mesenteric artery and the coeliac axis are cut between double ligatures close to the abdominal aorta. The hepatic portal vein is ligated and cut in the same manner. After ligating and cutting the esophagus and the rectum, the abdominal portion of the gastrointestinal tract is removed, together with the pancreas and the spleen. The muscle and the skin incisions are sutured. After completion of the operation the rats are left for 15 min to equilibrate.

Drugs are injected intravenously through the venous cannula. Blood samples are withdrawn from the aortic cannula before and at various intervals after drug administration to be assayed for insulin, blood glucose and non-esterified fatty acids.

EVALUATION

All results are expressed as mean ±SEM. Statistical analysis is performed using ANOVA.

CRITICAL ASSESSMENT OF THE METHOD

The described "functional" evisceration can be performed within 2–3 min without any hemorrhage or any signs of shock, even in hypophysectomized or adrenalectomized animals. That the liver remaining *in situ* is effectively cut off from the blood stream is proven by the fact that rats eviscerated in this way will die within 1–2 h without infusion of glucose.

MODIFICATIONS OF THE METHOD

Some authors removed also the kidneys and the adrenals in rats (Creutzfeldt and Deuticke 1962).

Dogs were eviscerated and nephrectomized under sodium pentobarbital anesthesia by Levine et al. (1950).

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J.9.2 Evisceration in rabbits

PURPOSE AND RATIONALE

Evisceration in rabbits was performed by Drury (1935), Wick and Drury (1963), Lippmann and Hommel (1968, 1969), Menzel and Haupt (1972). A detailed description of the technique was given by Lippmann and Hommel (1968).

PROCEDURE

Rabbits of both sexes weighing 2.6–3.2 kg kept on standard diet and tap water ad libitum are used. Food is withdrawn 12 h before surgery and the abdominal skin

is shaved. The animals are anesthetized with 50 mg/kg hexobarbital sodium intravenously. After fixation of the animal, the abdominal cavity is opened by electrocautery from the xyphoid process until 3 cm cranial of the symphysis. The rectum is cut between double ligatures as far caudally as possible. Bleeding from the arteria rectalis has to be avoided. The arteria mesenterica inferior, which is a tiny vessel in the rabbit, is ligated close to the aorta. The root of the mesentery is cut up to the superior mesenteric artery which is tightly ligated. Then the arteria coeliaca is ligated in the same manner allowing complete removal of the root of the mesentery. The esophagus is ligated close to the cardia. After double ligature of the portal vein the whole gastrointestinal tract including the pancreas and the spleen is isolated and the liver is functionally cut off. To eliminate the function of the kidneys, the renal arteries are ligated close to the aorta and the ureters close to the hilus. Cannulae are introduced into the caval vein via the renal veins. The right renal vein is ligated close to the hilus and the tip of a polyethylene catheter placed 4–6 cm caudal from the inferior caval vein for blood withdrawal; the left renal vein is also ligated close to the hilus and the tip of a polyethylene catheter placed 6 cm cranial from the inferior caval vein for infusion.

The animal receives via the catheter 750 IU heparin, 0.3 mg/kg lobeline hydrochloride, 3 mg/kg pentylenetetrazole, 0.01 mg/kg ouabain, and 1.3 mg/kg synephrine to stabilize the cardiovascular function.

Drugs are injected intravenously through the infusion cannula. Blood samples are withdrawn from the other cannula before and at various intervals after drug administration to be assayed for insulin and blood glucose.

EVALUATION

All results are expressed as mean ±SEM. Statistical analysis is performed using ANOVA.

MODIFICATIONS OF THE METHOD

Schäfer (1990) measured glucose disappearance rates into peripheral tissues *in vivo* in adult eviscerated, nephrectomized and adrenalectomiced rabbits, Wistar rats and non-diabetic sand rats with physiological insulin concentrations in the blood.

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Chapter K
Antidiabetic activity[1]

[1] Contribution of the biochemical part by G. Müller, Review by A. W. Herling.

K.1 Methods to induce experimental diabetes mellitus

K.1.0.1 Pancreatectomy in dogs

GENERAL CONSIDERATIONS

Dysfunction of the visceral tract has been considered for a long time to be the cause of diabetes mellitus. Bomskov (1910) reported severe diabetic symptoms in dogs after cannulation of the ductus lymphaticus. This observation, however, could not be confirmed in later experiments (Vogel 1963). The technique was similar to that described by Gryaznova (1962, 1963) for ligation of the thoracic duct in dogs.

Von Mehring and Minkowski (1890) noted polyuria, polydipsia, polyphagia, and severe glucosuria following removal of the pancreas in dogs. The final proof for the existence of a hormone in the pancreas was furnished by Banting and Best (1922) who could reduce the elevated blood sugar levels in pancreatectomized dogs by injection of extracts of the pancreatic glands. The role of the pituitary gland in development of diabetes has first been elucidated by Houssay (1930, 1931) in pancreatectomized dogs (Survey by Beyer and Schöffling 1986).

PURPOSE AND RATIONALE

The technique of complete pancreatectomy in the dog as described in detail by Foà (1971) and by Sirek (1986) has been used by many scientists as a relevant animal model for diabetes mellitus in man. Some remarks of our own experiences are added to an abbreviated version of Sirek's description.

PROCEDURE

Male Beagle dogs weighing 12–16 kg are used. The animal is anesthetized with an intravenous injection of 50 mg/kg pentobarbital sodium and placed on its back. After removal of the fur and disinfection of the skin a midline incision is made from the xyphoid process reaching well below the umbilicus. Bleeding vessels are ligated and the abdomen is entered through the linea alba. The falciform ligament is carefully removed and the vessels ligated. A self-retaining retractor is applied. By passing the right hand along the stomach to the pylorus, the duodenum with the head of the pancreas is brought into the operating field. First, the mesentery at the unicate process is cut and the process itself is dissected free. The glandular tissue is peeled off from the inferior pancreatico-duodenal artery and vein. The vessels themselves are carefully preserved.

Along a line of cleavage which exists between the pancreas, the pancreaticoduodenal vessels and the duodenal wall, the pancreas is separated from the duodenum and from the carefully preserved pancreaticoduodenal vessels. The small vessels to the pancreas are ligated. The dissection is carried out from both sides of the duodenum. In the area of the accessory pancreatic duct the glandular tissue being attached very firmly has to be carefully removed in order to leave no residual pancreatic tissue behind.

The pancreatic duct is cleaned, doubly ligated and cut between the ligatures. The dissection proceeds until one encounters a small lobe containing the main pancreatic duct. The glandular tissue adheres here firmly to the duodenum. Blunt dissection and ligation of the vessels is followed by ligation of the pancreatic duct. By pulling on the pylorus and the stomach, the pyloric and the splenic parts of the pancreas are delivered into the wound. The duodenal part is placed back into the abdominal cavity. The mesentery of the body and tail of the pancreas is cut with scissors. The small vessels are doubly ligated and cut. The pancreatic tissue is bluntly dissected from the splenic vessels. The pancreatic branches of the splenic vessels are doubly ligated and cut. Working in direction from the spleen to the pylorus, the pyloric part of the pancreas is the last one to be dissected. Finally, all pancreatic tissue is removed.

The surgical field is checked once more for pancreatic remnants. The concavity of the duodenum and its mesentery is approximated by a few silk stitches and the omentum is wrapped around the duodenum. Retroperitoneal injection of 5 ml 1% procaine solution is given to prevent intussusception of the gut. 250 000 IU penicillin G in saline solution are instilled into the peritoneal cavity. The abdominal wall and the subcutaneous layer are closed by sutures and finally the skin is sutured with continuous everting mattress stitches.

After the operation, the animal receives via a jugular vein catheter for 3–4 days the following treatment: 1 000 ml 10% glucose solution with 10 IU human insulin Regular®, 3 ml 24% Borgal® (sulfadioxin/trimethoprim) solution, 2 ml 50% metamizol and 400 IU secretin. On the third day, the animal is offered milk. After the animal has passed the first milk feces, it is given daily dry food (e.g. Vipromix®, Nagut, Laage, Germany) together with a preparation of pancreatic enzymes (e.g. Viveral® or Festal®, Hoechst AG, Germany). Insulin is substituted with a single daily subcutaneous dose of 34 IU Retard-Insulin (e.g. Ultratard® HM). Vitamin D_3 is given every three months as a intramuscular injection of 1 ml Vigantol® forte.

MODIFICATIONS OF THE METHOD

Experiments performed by Houssay (1930, 1931) performing hypophysectomy in pancreatectomized dogs revealed amelioration of the diabetic state. These experiments contributed to the understanding of hormonal control in diabetes mellitus.

Rappaport et al. (1966), Lau et al. (1976) used a pedunculated subcutaneous autotransplant of an isolated pancreas remnant for the temporary deprivation of internal secretion in the dog.

Subtotal pancreatectomy in **rats** was described by Scow (1957), Scow et al. (1957), Wagner and Cardeza (1957), Bonner-Weir et al. (1983), Noguchi et al. (1994), Tanigawa et al. (1997). The pancreatic tissue between the common bile duct, duodenal loop, and portal vein in the duodenal segment, along the greater curvature in the gastric segment, and along the splenic vein in the splenic segment, was surgically excised in 3- to 4-weeks old rats weighing 80–100 g.

Greeley (1937) proposed a 3-stage procedure with 3–4 weeks intervening between operations for pancreatectomy in **rabbits**.

Itoh and Maki (1996) reported surgical removal of 90% of pancreatic tissue in 7 or 13 weeks old **mice**. Under sodium pentobarbital anesthesia (65 mg/kg i.p.), the pancreas and the spleen were surgically removed with careful conservation of the common bile duct and major vessels surrounding the duodenum. Approximately 10% (by weight and by insulin content) of the pancreas tissue was left intact adjacent to the lower duodenal loop.

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K.1.0.2 Alloxan induced diabetes

PURPOSE AND RATIONALE

Surveys on chemically induced diabetes in animals were given by Frerichs and Creutzfeldt (1968, 1971).

Hyperglycemia and glucosuria after administration of alloxan has been described in several species, such as in dogs (Brunschwig et al. 1943; Tasaka et al. 1988), in rabbits (Baily and Baily 1943), in rats (Dunn and McLetchie 1943; Goldner and Gomori 1944) and in other species (Frerichs and Creutzfeldt 1968, 1971). Guinea pigs have been found to be resistant (Maske and Weinges 1957). Dosage and treatment regimen have been elaborated for the most frequently used species. In most species a triphasic time course is observed: an initial rise of glucose is followed by a decrease, probably due to depletion of islets from insulin, again followed by a sustained increase of blood glucose.

PROCEDURE

Rabbits weighing 2.0 to 3.5 kg are infused via the ear vein with 150 mg/kg alloxan monohydrate (5.0 g/100 ml, pH 4.5) for 10 min resulting in 70% of the animals to become hyperglycemic and uricosuric. The rest of the animals either die or are only temporarily hyperglycemic (Baily and Baily 1943; Pincus et al. 1954; Bänder et al. 1969).

Rats of Wistar or Sprague-Dawley strain weighing 150–200 g are injected subcutaneously with 100–175 mg/kg alloxan (Blum and Schmid 1954; Katsumata and Katsumata 1990; Katsumata et al. 1993).

Male **Beagle dogs** weighing 15–20 kg are injected intravenously with 60 mg/kg alloxan. Subsequently, the animals receive daily 1 000 ml 5% glucose solution with 10 IU Regular insulin for one week and canned food ad libitum. Thereafter, a single daily dose of 28 IU insulin (Ultratard HM®) is administered subcutaneously (Brunschwig at al 1943; Geisen 1988).

MODIFICATIONS OF THE METHOD

Kodoma et al. (1993) described a new diabetes model induced by neonatal alloxan treatment in rats. Male Sprague Dawley rats 2, 4, or 6 days of age were injected intraperitoneally with 200 mg/kg of alloxan monohydrate after a 16 h fast. The most severe diabetic symptoms occurred in rats injected on day 6.

Keikkila et al. (1974) reported the prevention of alloxan-induced diabetes by ethanol administration in mice.

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K.1.0.3 Streptozotocin induced diabetes

PURPOSE AND RATIONALE

Rakieten et al. (1963) reported the diabetogenic activity of the antibiotic streptozotocin. The compound turned out to be specifically cytotoxic to beta-cells of the pancreas.

PROCEDURE

Male Wistar rats weighing 150–220 g fed with a standard diet are injected with 60 mg/kg streptozotocin (Calbiochem) intravenously. As with alloxan, three phases of blood glucose changes are observed. Initially, blood glucose is increased, reaching values of 150–200 mg% after 3 h. Six–eight h after streptozotocin, the serum insulin values are increased up to 4 times, resulting in a hypoglycemic phase which is followed by persistent hyperglycemia. Severity and onset of diabetic symptoms depend on the dose of streptozotocin. After the dose of 60 mg/kg i.v., symptoms occur already after 24–48 h with hyperglycemia up to 800 mg%, glucosuria and ketonemia. Histologically, the beta-cells are degranulated or even necrotic. A steady state is reached after 10–14 days allowing to use the animals for pharmacological tests.

CRITICAL ASSESSMENT OF THE METHOD

Streptozotocin induced diabetes in laboratory animals, mostly in rats, has become a valuable tool in diabetes research being used by many investigators.

MODIFICATIONS OF THE METHOD

A survey on susceptibility of various species to streptozotocin was given by Frerichs and Creutzfeldt (1971).

Multiple low doses of streptozotocin induce immune pancreatic insulitis in rats thereby mimicking immune type 1 diabetes in humans (Like and Rossini 1976; Rossini et al. 1977).

Miller (1990) described the effect of streptozotocin on the **golden Syrian hamster** using a single i.p injection of 50 mg/kg streptozotocin.

Enhancement of streptozotocin induced diabetes in **CD-1 mice** by cyclosporin A was reported by Iwakiri et al. (1987).

Grussner eat al (1993) induced long-lasting diabetes mellitus in **Yorkshire Landrace pigs** with a dosage of 150 mg/kg streptozotocin.

Stosic-Grujicic et al. (1999) described protection of **mice** from multiple low dose streptozotocin-induced insulitis and diabetes by the immunosuppressive drug leflunomide.

Bleich et al. (1999) found that elimination of leukocyte 12-lipoxygenase in mice ameliorates low dose streptozotocin-induced diabetes by increasing islet resistance to cytokines and decreasing macrophage production of nitric oxide.

Masutani et al. (1998) studied the role of poly(ADP-ribose)-polymerase (Parp) in streptozotocin-induced diabetes. Parp-deficient ($Parp^{-/-}$) mice were established by disrupting *Parp* exon 1 using the homologous recombination technique. These mice were almost resistant to streptozotocin-induced diabetes.

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K.1.0.4 Other diabetogenic compounds

PURPOSE AND RATIONALE

Several other compounds have been found to induce symptoms of diabetes and/or obesity, such as dithizone (Maske and Weinges 1957; Frerichs and Creutzfeldt 1971; Hansen et al. 1989; Goldberg et al. 1991) or goldthioglucose (Stauffacher et al. 1967; Caterson et al. 1988; Silva and Hernandez 1989; Heydrick et al. 1995) or monosodium glutamate (Sartin et al. 1985).

PROCEDURE

Goldberg et al. (1991) injected various chelators, such as dithizone, 8-(p-toluene-sulfonylamino)-quinoline (8-TSQ), and 8-(benzenesulfanylamino)-quinoline (8-BSQ) in a single i.v. dose of 40–100 mg/kg to cats, rabbits, golden hamsters and mice. Dithizone injection causes a triphasic glycemic reaction in rabbits. A phase of initial hyperglycemia is detected after 2 h, followed by a normoglycemic phase after 8 h and a secondary permanent hyperglycemic phase after 24–72 h. Histologically, complete and partial degranulation of beta cells is observed.

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K.1.0.5 Hormone induced diabetes

Growth hormone induced diabetes

Cotes et al. (1949) described the diabetogenic action of pure anterior pituitary growth hormone in cats. In intact adult dogs and cats the repeated administration of growth hormone induces an intensively diabetic condition with all symptoms of diabetes including severe ketonuria and ketonemia. Rats of any age subjected to a similar treatment do not become diabetic but grow faster (Young 1945) and show striking hypertrophy of the pancreatic islets.

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Corticosteroid induced diabetes

Ingle (1941) described hyperglycemia and glucosuria in forced fed rats treated with cortisone. In the guinea pig and in the rabbit, experimental corticoid diabetes could be obtained without forced feeding (Hausberger and Ramsay 1953; Abelove and Paschkis 1954). In the rat, the adrenal cortex, stimulated by corticotrophin, has the capacity to secrete amounts of steroids which induce steroid diabetes (Ingle et al. 1946).

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K.1.0.6 Insulin deficiency due to insulin antibodies

PURPOSE AND RATIONALE

A transient diabetic syndrome can be induced by injection of guinea pig anti-insulin serum in various species (Moloney and Coval 1955; Wright 1968).

PROCEDURE

Bovine insulin, dissolved in acidified water (pH 3.0), is incorporated in a water-oil emulsion based on complete Freund's adjuvant or a mixture of paraffin oil and lanolin. A dose of 1 mg insulin is injected in divided doses subcutaneously to male guinea pigs weighing 300–400 g. Injections are given at monthly intervals and the guinea pigs are bled by cardiac puncture two weeks after the second and subsequent doses of antigen. It is possible to get 10 ml blood from every animal once a month.

Intravenous injection of 0.25–1.0 ml guinea pig anti-insulin serum to rats induces a dose-dependent increase of blood glucose reaching values up to 300 mg%. This effect is unique to guinea pig anti-insulin serum and is due to neutralization by insulin antibodies of endogenous insulin secreted by the injected animal. In this way a state of insulin deficiency is induced. It persists as long as antibodies capable of reacting with insulin remain in the circulation. Slow rate intravenous infusion or intraperitoneal injection prolongs the effect for more than a few hours. However, large doses and prolonged administration accompanied by ketonemia, ketonuria, glucosuria, and acidosis are fatal to the animals. After lower doses, the diabetic syndrome is reversible after a few hours.

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K.1.0.7 Virus induced diabetes

Juvenile-onset (type I) diabetes mellitus may be due to virus infections and β-cell specific autoimmunity (Craighead 1978). The D-variant of encephalomyocarditis virus (EMC-D) selectively infects and destroys pancreatic β-cells in susceptible mouse strains similar to human insulin-dependent diabetes (Yoon et al. 1980; Giron and Patterson 1982; Giron et al. 1983; Vialettes et al. 1983). Adult, male ICR Swiss mice are susceptible to the diabetogenic effect of the D-variant of encephalomyocarditis virus in contrast to adult C3H/HeJ male mice which are relatively resistant. Pretreatment with cyclosporin A, a potent immunosuppressive drug, results in increased severity and incidence of diabetes in susceptible ICR Swiss mice and induction of diabetes in resistant C3H/HeJ mice (Gould et al. 1985).

MODIFICATIONS OF THE METHOD

Hirasawa et al. (1997) studied the possible role of macrophage-derived soluble mediators in the pathogenesis of encephalomyelitis virus-induced diabetes in **mice**. The inactivation of macrophages prior to viral infection resulted in the prevention of diabetes.

Utsugi et al. (1992) demonstrated that intraperitoneal inoculation with NDK25, a variant of encephalomyocarditis virus which has been cloned from the M variant of encephalomyocarditis virus, caused DBA/2 mice to develop non-insulin-dependent diabetes mellitus.

See and Tilles (1995) challenged CD-1 mice with a diabetogenic strain (E2) of coxsackievirus B4. Islet cell destruction was associated with chronic islet cell inflammation, elevation of islet cell antibody, and prolonged presence of viral RNA in the pancreas.

Stubbs et al. (1994) investigated the effect of Kilham rat virus (KRV) infection on GLUT2 expression in diabetes-resistant BB/Wor **rats**. Viral antibody-free diabetes resistant rats did not develop spontaneous diabetes, but inoculation with Kilham rat virus induced autoimmune beta cell-destruction and hyperglycemia.

Hayashi et al. (1995) investigated the role of adhesion molecules in the reovirus type2-induced diabetes-like syndrome in mice.

Ellerman et al. (1996) studied Kilham rat virus induced autoimmune diabetes in multiple strains of rat.

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K.2 Genetically diabetic animals

K.2.0.1 General considerations

Several animal species, mostly rodents, were described to exhibit spontaneously diabetes mellitus on a hereditary basis. These findings were highly appreciated with the expectation to get more insight into the pathogenesis of diabetes in humans. During the last few years since the discovery of leptin (Zhang et al. 1994) and its downstream signal transduction cascade (Friedman and Halaas 1998) tremendous new insight of the genetics of diabetic and obese animal disease models derived. Up to now at least 6 genetically diabetic animal models exhibit defects in the leptin pathway: The *ob* mutation in the mouse resulted in leptin deficiency. The *db* mutation in the mouse and the *cp* and *fa* mutations in the rat are different mutations of the leptin receptor gene. The *fat* mutation in the mouse results in a biologically inactive carboxipeptidase E, which processes the prohormone conversion of POMC into α-MSH, which activates the hypothalamic MC4 receptor. Finally the Agouti yellow (*y*) mouse exhibit an ubiquitous expression of the Agouti protein which represents an antagonist of the hypothalamic MC4 receptor.

Symptoms of diabetes and obesity are overlapping in many animal models (see also L.2. Genetically obese animals).

CRITICAL ASSESSMENT

The pathophysiological mechanisms which finally lead to the diabetes phenotype (hyperglycemia, hyperinsulinemia and insulin resistance) exhibited of the various animal disease models for non-insulin dependent diabetes do not necessarily be identical to those in human disease. Therefore, detailed knowledge about the (patho-)physiology of these animal disease models is a prerequisite for interpretation of experimental results and their value for the human disease.

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K.2.0.2 Spontaneously diabetic rats

The occurrence of spontaneous diabetes has been reported in several strains of rats:

BB RAT

The BB rat (Bio Breeding (BB) rat) is a model of spontaneous diabetes associated with insulin deficiency and insulitis due to autoimmune destruction of pancreatic beta cells. (Nakhooda et al. 1977, 1978, Like et al. 1982; Oschilewski et al. 1985; Lee et al. 1988; Solomon et al. 1989; Papaccio and Mezzogiorno 1989; Kolb et al. 1990; Velasquez et al. 1990; Lefkowith et al. 1990; Gottlieb et al. 1990; Ellerman et al. 1993). Diabetes is inherited as an autosomal recessive trait and develops with equal frequency and severity among males and females. The onset of clinical diabetes is sudden, and occurs at about 60–120 days of age. Within several days, diabetic animals are severely hyperglycemic,hypoinsulinemic, and ketotic unless insulin treatment is instituted.

Pipeleers et al. (1991) described the transplantation of purified islet cells in diabetic BB rats.

Hao et al. (1993) reported that the immunosuppressive agent mycophenolate mofetil can prevent the development of diabetes in BB rats.

Klöting and Vogt (1991) characterized the features of a subline of diabetes-prone BB rats (BB/OK rats). The circadian variations in blood pressure and heart rate of this strain were compared with spontaneously hypertensive rats (Berg et al. 1997).

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WBN/KOB RAT

Spontaneous hyperglycemia, glucosuria and glucose intolerance have been observed in aged males of an inbred Wistar strain, named the WBN/Kob rat (Nakama et al. 1985; Tsuchitani et al. 1985; Koizumi 1989; Shimoda et al. 1993).These animals exhibit impaired glucose tolerance and glucosuria at 21 weeks of age. Obvious decreases in the number and size of islets are found already after 12 weeks of age. Fibrinous exudation and degeneration of pancreatic tissue are observed in the exocrine part, mainly around degenerated islets and pancreatic ducts in 16 weeks old males. These rats develop demyelinating, predominantly motor neuropathy, later accompanied by axonal changes (Yagihashi et al. 1993).

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COHEN DIABETIC RAT

Diabetes in Cohen rats is characterized by hyperglycemia, glucosuria, and hyperinsulinemia, with late development of hypoinsulinemia, insulin resistance, and a decrease in the number and sensitivity of insulin receptors. The rats develop overt diabetes and diabetes related complications when fed a diet rich in sucrose or other refined sugars and poor in copper content, but not when fed a starch or stock diet (Cohen 1972; Velasquez et al. 1990).

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GOTO-KAKIZAKI RAT

Non-obese, insulin-resistant Goto-Kakizaki (GK) rats are a highly inbred strain of Wistar rats that spontaneously developed type II diabetes. Defects in glucose-stimulated insulin secretion, peripheral insulin resistance, and hyperinsulinemia are seen as early as 2 to 4 weeks after birth. Impaired skeletal muscle glycogen synthase activation by insulin was observed, accompanied by chronic activation of diacylglycerol-sensitive protein kinase C.

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ZUCKER-FATTY RAT

The Zucker-fatty rat is a classic model of hyperinsulinemic obesity. (Zucker 1965). Obesity is due to a simple autosomal recessive (*fa*) gene and develops at an early age. Obese Zucker rats manifest mild glucose intolerance, hyperinsulinemia, and peripheral insulin resistance similar to human NIDDM. However, their blood sugar level is usually normal throughout life (Bray 1977; Clark et al. 1983; McCaleb and Sredy 1992; Abadie et al. 1993; Alamzadeh et al. 1993; Kasim et al. 1993; Galante et al. 1994).

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ZUCKER DIABETIC FATTY RAT (ZDF/DRT-FA)

The obese Zucker Diabetic Fatty rat originally derived from the Zucker fatty rat (Peterson et al. 1990). This strain develops diabetes with hyperglycemia of about 20 mmol/l. The males and females become diabetic at the age of 6 to 8 weeks and 9 to 11 weeks, respectively. Diabetes phenotype develops due to lipotoxicity to

the β-cell (Lee et al. 1994). These rats are characterized besides hyperglycemia by insulin resistance, moderate hyperinsulinemia, extreme hyperphagia due to the loss of calories by glucosuria and obesity.

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WDF/TA-FA RAT

The WDF/Ta-fa rat, commonly referred to as the Wistar fatty rat, is a genetically obese, hyperglycemic rat established by the transfer of the fatty *(fa)* gene from the Zucker rat to the Wistar Kyoto rat. (Ikeda et al. 1981; Kava et al. 1989; Velasquez et al. 1990). The Wistar fatty rat exhibits obesity, hyperinsulinemia, glucose intolerance, hyperlipidemia, and hyperphagia similar to Zucker rats being, however, more glucose intolerant and insulin resistant than Zucker rats. Hyperglycemia is usually not observed in females but can be induced by addition of sucrose to the diet.

Kobayashi et al. (1992) found an increase of insulin sensitivity by activation of insulin receptor kinase by Pioglitazone in Wistar fatty rats (*fa/fa*).

Sugiyma et al. (1992) found a reduction of glucose intolerance and hypersecretion of insulin in Wistar fatty rats after treatment with pioglitazone for 10 days.

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OLETF RAT

A spontaneously diabetic rat with polyuria, polydipsia, and mild obesity was discovered in 1984 in an outbred colony of Long-Evans rats. A strain of rats developed from this rat by selective breeding has since been maintained at the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) and named OLETF. The characteristic features of OLETF rats are: (1) late onset of hyperglycemia (after 18 weeks of age), (2) a chronic course of disease, (3) mild obesity, (4) inheritance by males, (5) hyperplastic foci of pancreatic islets, and (6) renal complications (nodular lesions). The clinical and pathological features of disease in OLETF rats resemble those of human NIDDM.

Administration of diazoxide (0.2% in diet), an inhibitor of insulin secretion, to OLETF rats from the age of 4 to 12 weeks completely prevented the development of obesity and insulin resistance (Aizawa et al. 1995).

Ishida et al. (1995) found that insulin resistance preceded impaired insulin secretion in OLETF rats.

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ESS-RAT

The occurrence of spontaneous diabetes in a colony of rats (Stilman Saldago), called eSS-rat was reported by Tarrés et al. (1981). The animals show abnormal glucose tolerance tests from the age of 2 months onwards. The syndrome consists of a mild type of diabetes that does not diminish the longevity of the animals. Six-months old rats show disruption of the islet architecture and fibrosis of the stroma (Dumm et al. 1990).

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OBESE SHR RAT

The strain of obese SHR rats was developed by Koletsky (1973, 1975) by mating a spontaneous hypertensive female rat of the Kyoto-Wistar strain with a normotensive Sprague Dawley male. After several generations of selective inbreeding, these obese SHR exhibited obesity, hypertension, and hyperlipidemia. In addition, some rats developed hyperglycemia and glucosuria associated with giant hyperplasia of pancreatic islets.

From these rats, several substrains were developed, such as the JCR:LA-corpulent rat which exhibits a syndrome characterized by obesity, hypertriglyceridemia and hyperinsulinemia with impaired glucose tolerance (Russell et al. 1994).

Reduced insulin receptor signaling was found in the obese spontaneously hypertensive Koletsky rat (Friedman et al. 1997).

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SHR/N-CP RAT

The congenic SHR/N-cp rat strain, developed at the National Institutes of Health, USA, (Hansen 1983; Michaelis et al. 1986; Hansen 1988; Adamo et al. 1989) was derived by mating a male Koletsky rat heterozygous for the corpulent gene (cp/+) to a female rat of the Okamoto strain. After a minimum of 12 backcrosses, homozygous (cp/cp) SHR/N-cp rats exhibit obesity, mild hypertension, hyperinsulinemia, and glucose intolerance.

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BHE RAT

The BHE rat colony was originally developed by breeding black and white hooded rats of the Pennsylvania State College strain and albino rats of the Yale (Osborne Mendell) strain. The BHE rat is a model in which the diabetic state is manifested only at maturity. BHE rats have hyperinsulinemia at 50 days of age associated with glucose intolerance and tissue resistance to insulin. Later on, BHE rats have less hyperinsulinemia with reduced pancreatic insulin stores, and show mild hyperglycemia and hyperlipidemia.

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K.2.0.3 Spontaneously diabetic mice

KK MOUSE

Nakamura (1962, 1967) reported on a diabetic strain of the KK-mouse. The animals were moderately obese and showed polyphagia and polyuria. Mice at the age of seven months or older showed glucosuria and blood sugar levels up to 320 mg%. The pancreatic insulin content was increased, but histologically degranulation of the beta-cells and hypertrophy of the islets were found. Sections of the liver showed a reduction of glycogen and an increase in lipid content.

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KK-A^{Y} MOUSE

Iwatsuka et al. (1970) reported on yellow KK mice (also named KK-A^{y} mice), carrying the yellow obese gene (A^{y}). These mice develop marked adiposity and diabetic symptoms in comparison with their littermates, black KK mice. Blood glucose and circulating insulin levels as well as HbA_{1c} levels were increased progressively from 5 weeks of age. Degranulation and glycogen infiltration of B cells were followed by hypertrophy and central cavitation of islets. Lipogenesis by liver and adipose tissue were increased. Insulin sensitivity of adipose tissue was more remarkably reduced than in black KK mice to its complete loss at 16 weeks of age. Renal involvement is uniquely marked by early onset and rapid development of glomerular basement membrane thickening (Diani et al. 1987).

KK-A^{y} mice can be used to demonstrate the extrapancreatic action of antidiabetic drugs, such as glimepiride, a novel sulfonylurea (Satoh et al. 1994).

Sohda et al. (1990) evaluated ciglitazone and a series of 5-[4-(pyridylalkoxy)benzyl]-2,4-thiazolidinediones for hypoglycemic and hypolipemic activities in yellow KK mice.

Hofmann et al. (1992) evaluated the expression of the liver glucose transporter GLUT2 and the activity and the expression of phosphoenolpyruvate carboxykinase in the liver of obese KKA^{Y} mice after treatment with the oral antidiabetic agent pioglitazone.

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NOD MOUSE

The NOD mouse strain was established by inbreeding diabetic CTS mice derived originally from the JCL-ICR strain. Like the BB rat, the NOD mouse is a model of insulin dependent diabetes mellitus and develops hypoinsulinemia secondary to autoimmune destruction of pancreatic β cells in association with insulitis and auto antibody production. NOD mice develop diabetes abruptly between 100 and 200 days of age, as well as rapid weight loss, polyuria, polydipsia, and severe glucosuria. Without insulin treatment, they do not survive for more than one month and usually die from ketosis. The onset of diabetes can be prevented by an immunmodulating drug (Baeder et al. 1992) or by a soluble interleukin-1 receptor (Nicoletti et al. 1994).

Hutchings and Cooke (1995) compared the protective effects afforded by intravenous administration of bovine or ovine insulin to young NOD mice.

Bergerot et al. (1997) reported that feeding small amounts (2–20 μg) of human insulin conjugated to cholera toxin B subunit can effectively suppress β-cell destruction and clinical diabetes in adult NOD mice.

Insulin-dependent diabetes mellitus in NOD Mice is the result of a CD4^{+} and CD8^{+} T cell-dependent auto-immune process directed against the pancreatic β-cells (Serreze and Leiter 1994; Verdaguer et al. 1997).

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OBESE HYPERGLYCEMIC MICE

Ingalls et al. (1950), Mayer et al. (1951), Bleisch et al. (1952) observed hereditary diabetes in genetically obese mice. The obese hyperglycemic mice were glycosuric, the non-fasting blood sugar levels were about 300 mg%, but neither ketonuria nor coma were observed. One of the most interesting features was insulin-resistance; doses as high as 400 IU/kg had little effect on blood sugar. The serum insulin-like activity was high, the islands of Langerhans were hypertrophic, their insulin content was increased and the liver glycogen stores were decreased. Kidneys and other organs did not show pathological changes. Obviously, the diabetic condition of this and other strains of obese hyperglycemic mice is different from that of the human diabetic patient. The *ob* mutation was identified as a mutation in the leptin gene in adipose tissue (Zhang et al. 1994) and the substitution of leptin reverses the obese and diabetic phenotype completely (Halaas et al. 1995). Other strains or substrains of mice with obesity and hyperglycemia have been described by Dickie (1962), Westman (1968), Stein et al. (1970), Coleman and Hummel (1973), Herberg and Coleman (1977).

Gill and Yen (1991) studied the effect of ciglitazone on endogenous plasma islet amyloid polypeptide (amylin) and insulin sensitivity in obese-diabetic viable yellow mice (VY/Wfl-A^{vy}/a).

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DIABETES MOUSE (*DB/DB*)

The diabetes *db/db* mouse strain is derived from an autosomal recessive mutation having occurred spontaneously in mice of the C57BL/KsJ strain which was identified as a mutation in the leptin receptor gene (Tartaglia et al. 1995). On this basis, the diabetes mouse (C57BL/6J *db/db*) consistently develops a severe diabetic syndrome similar to that found in the C57BL/KsJ *ob/ob* mouse, characterized by early onset of hyperinsulinemia, and hyperglycemia up to 20 to 25 mmol/l. (Hummel et al. 1966; Coleman and Hummel 1967; Like et al. 1972). The *db/db* mouse, in contrast to the *ob/ob* mouse, develops significant nephropathy (Gardner 1978). Mutations on the leptin receptor result in an obese phenotype identical to that of *ob* mice (Li et al. 1998). C57BL/KsJ *ob/ob* mice are phenotypically the same as other strains of *db* mice. The leptin receptor (Ob-R) gene encodes 5 alternatively spliced forms, Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd (Lee et al. 1996). In the C57BL/KsJ *ob/ob* mouse strain, the Ob-Rb transcript contains an insert with a premature stop codon as a result if abnormal splicing (Chen et al. 1996; Friedman and Halaas 1998).

Coleman and Hummel (1969) joined adult diabetic mice (*db/db*) of the C57BL/Ks strain in parabiosis with normal mice of the same sex. Little, if any, amelioration of the disease was observed in parabiont diabetics and no symptoms of diabetes were observed in the normal parabiont. Instead, the normal partners lost weight, became hypoglycemic, and died of apparent starvation 50 days after surgery. In contrast, the diabetic partners gained weight rapidly and remained diabetic.

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DIABETES OBESITY SYNDROME IN CBA/CA MICE

A spontaneous maturity onset diabetes obesity syndrome occurs in a small proportion (10–20%) of male CBA/Ca mice. Inbreeding can increase the incidence to 80%. It occurs at 12–16 weeks of age, and is characterized by hyperphagia, obesity, hyperglycemia, hypertriglyceridemia, hyperinsulinemia, and an impaired glucose tolerance. The mice are also resistant to exogenous insulin. Female mice remain normal except for a slight increase in serum insulin. The male obese diabetic mice have a normal life expectancy.

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WELLESLEY MOUSE

The Wellesley mouse, described first by Jones (1964), is a hybrid with predisposition to diabetes mellitus. The diabetic animals have elevated levels of immunoreactive insulin in serum, enlarged pancreatic islets and reduced insulin responsiveness in peripheral tissues (Cahill et al. 1967; Gleason et al. 1967; Like and Jones 1967).

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K.2.0.4 Chinese hamster

Meier and Yerganian (1959, 1961) described the occurrence of hereditary diabetes mellitus in the Chinese

hamster (*Cricetulus griseus*). Blood sugar levels of diabetic hamsters were elevated from a normal of 110 mg% up to 600 mg%. Severe polyuria, glucosuria, ketonuria, and proteinuria were observed. The diabetic condition could be improved by administration of insulin, and oral antidiabetic drugs were effective in mildly diabetic hamsters. Pathological changes were seen in histological sections of pancreas, liver and kidney. The number of pancreatic islets was decreased, and the cells of the remaining islets were abnormal. This animal model has been studied by several authors.

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K.2.0.5 Other species with inherited diabetic symptoms

SAND RAT

The sand rat (*Psammomys obesus*) lives in the desert regions of North Africa and the Near East. In the laboratory the animals develop diabetic symptoms when fed Purina laboratory chow instead of an all vegetable diet (Hackel et al. 1965, 1967; Miki et al. 1967; deFronzo et al. 1967; Brodoff et al. 1967; Strasser 1968). The diabetic syndrome in the sand rat usually develops within 2–3 months with variations in severity between the animals. Severely hyperglycemic animals die prematurely from ketosis. Initially, the pancreatic islets appear normal. In the intermediate stage of the disease, degranulation of pancreatic β cells is observed. This is followed by β cell degeneration and necrosis with resultant insulinopenia and ketonuria.

Histological studies by Dubault et al. (1995) showed insulitis in animals who became insulin-dependent in later stages and recommended *Psammomys obesus* as a model of latent IDDM in NIDDM patients.

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SPINY MOUSE

The spiny mouse (*Acomys cahirinus*) is a small rodent living in the semi-desert areas of the Eastern Mediterranean (Pictet et al. 1967). Diabetes occurs in about 15% of the animals under laboratory conditions accompanied by hyperplasia of the endocrine pancreas. Great variations in the appearance and severity of diabetes and obesity occur in this species. Some animals show obesity, mild hyperglycemia, and hyperinsulinemia. Others have frank hyperglycemia with glucosuria that leads to fatal ketosis. Regardless of the stage of the disease, all spiny mice characteristically have massive hyperplasia of pancreatic islets and increased pancreatic insulin content. Despite the large insulin stores, plasma insulin response to glucose is delayed or impaired suggesting an impairment of the insulin release mechanism.

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AFRICAN HAMSTER (*MYSTROMYS ALBICAUDATUS*)

Spontaneous diabetes mellitus was described in South African hamsters (*Mystromys albicaudatus*) by Packer et al. (1970), Stuhlman et al. (1972, 1974, 1975), Schmidt et al. (1974). Characteristics established as part of the diabetic syndrome in this species include hyperglycemia, glucosuria, ketonuria, polyuria, polyphagia and polydipsia. Pancreatic lesions include β-cell vacuolization, glycogen infiltration, nuclear pycnosis, margination of organelles, and β-cell death.

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TUCO-TUCO

The diabetic syndrome in Tuco-tucos (*Ctenomis talarum*) is similar to that in sand rats and spiny mice (Wise et al. 1972). However, Tuco-tucos tend to have less hyperglycemia and are less prone to ketosis. Many animals, mainly males, become hyperphagic and quite obese. Degranulation of β cell is the usual lesion in the pancreas, but amyloid hyalinization of islets has been observed in a few animals.

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MACACA NIGRA

A high incidence of spontaneous diabetes mellitus was found in *Macaca nigra* (Celebes black apes) with analogies to human diabetes (Howard 1972, 1974a,b, 1975). Abnormal signs include hyperglycemia, decreased clearance of glucose, in intravenous tolerance tests, reduced insulin secretion and increased serum lipids. Insulin secretory capacity is lost concomitant with amyloid infiltration into the islets of Langerhans. Secondary manifestations are atherosclerosis, thickened basement membranes of muscle capillaries, and cataracts. The genetic predisposition in these monkeys is exacerbated by changes in diet and environment.

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K.2.0.6 Transgenic animals

TRANSGENIC MICE

Schaefer et al. (1994) described a transgenic mouse model of chronic hyperglycemia. The extracellular ligand binding domain of the human insulin receptor can be expressed as a stable soluble protein that is efficiently secreted and binds insulin with high affinity. Expression under the mouse transferrin promoter of a transgene encoding a secreted derivative of the human insulin receptor in transgenic mice results in the accumulation of this high-affinity insulin-binding protein in the plasma. Alterations of glucose homeostasis are induced including postabsorptive hyperglycemia concomitant with increased hepatic glucose production and hyperinsulinemia.

Palmiter et al. (1987) developed a method of deleting specific cell lineages that entailed microinjection into fertilized eggs of a chimeric gene in which a cell-specific enhancer/promoter is used to drive the expression of a toxic gene product. Microinjection of a construct in which the elastase I promoter /enhancer is fused to a gene for diphtheria A polypeptide resulted in birth of mice lacking a normal pancreas because of the expression of the toxin in pancreatic acinar cells. A small pancreatic rudiment, containing islet and duct-like cells, was observed in some of the transgenic mice.

Aichele et al. (1994) used a synthetic peptide corresponding to an immunodominant epitope of lymphocytic choriomeningitis virus glycoprotein (LCMV GP) to prime or to tolerize $CD8^+$ T cells *in vivo*. Peptide-specific tolerance was then examined in transgenic mice expressing LCMV GP in the β islet cells of the pancreas. These mice developed $CD8^+$ T cell-mediated diabetes within 8–14 days after LCMV infection. Specific peptide-induced tolerance prevented autoimmune destruction of β islet cells and diabetes in this transgenic mouse model.

Oldstone et al. (1991) showed that virus infection triggers insulin-dependent diabetes mellitus in a transgenic mouse model.

Ablation of tolerance and induction of diabetes by virus infection in viral antigen transgenic mice was reported by Ohashi et al. (1991).

Von Herrath et al. (1994) investigated how virus induces a rapid or slow onset insulin-dependent diabetes mellitus in two distinct transgenic mouse models.

Von Herrath et al. (1995) evaluated the role of the costimulatory molecule B7-1 in overcoming peripheral ignorance in transgenic mice which expressed the glycoprotein or nucleoprotein of lymphocytic choriomeningitis virus as the self-antigen in pancreatic β-cells.

Von Herrath and Holz (1997) reported that pathological changes in the islet milieu precede infiltration of islets and destruction in β-cells by autoreactive lymphocytes in a transgenic model of virus-induced IDDM. RIP-LCMV transgenic mice that express the viral glycoprotein or nucleoprotein from lymphocytic choriomeningitis virus (LCMV) under control of the rat insulin promoter (RIP) in pancreatic β-cells develop autoimmune diabetes after infection with LCMV. Upregulation of MHC class II molecules associated with the attraction/activation of antigen presenting cells to the islets occurs as soon as 2 days after LCMV inoculation of transgenic mice, clearly before $CD4^+$ and $CD8^+$ lymphocytes are found entering the cells. Possibilities of treatment of virus-induced autoimmune diabetes were discussed (von Herrath et al. 1997).

Hebert et al. (1996) created transgenic mice to study the influence of overexpression of glutamine:fructose-6-phosphate amidotransferase on insulin resistance.

Moritani et al. (1996) reported the prevention of adoptively transferred diabetes in nonobese diabetic mice with IL-10-transduced islet-specific Th1 lymphocytes as a gene therapy model for autoimmune diabetes.

Birk et al. (1996) generated transgenic NOD mice carrying a murine Hsp60 transgene driven by the $H°2E_{\alpha}$ class II promoter in order to examine the hypothesis of a pathogenic role for self-reactive cells against the stress protein Hsp60 in autoimmune destruction of pancreatic cells in the diabetes of NOD mice.

Terauchi et al. (1997) reported development of non-insulin-dependent diabetes mellitus in double knockout mice with disruption of insulin receptor substrate-1 and β-cell glucokinase genes.

Insulin resistance and hyperinsulinemia in insulin receptor substrate-1 knockout mice was discussed by Jenkins and Storlien (1997).

Ueki et al. (2000) could restore insulin-sensitivity in IRS-1-deficient mice by adenovirus-mediated gene therapy.

Brüning et al. (1997) developed a polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles. Mice double heterozygous for null alleles in the insulin receptor and insulin receptor substrate-1 genes exhibit the expected ~50% reduction in expres-

sion of these two proteins, but a synergism at the level of insulin resistance with 5- to 50-fold elevated plasma insulin levels and comparable levels of β-cell hyperplasia.

Withers et al. (1998) showed that disruption of IRS-2 impairs both peripheral insulin signaling and pancreatic β-cell function. IRS-2-deficient mice showed progressive deterioration of glucose homeostasis because of insulin resistance in the liver and skeletal muscle and a lack of β-cell compensation for this insulin resistance.

Kulkarni et al. (1999) reported that in mice a tissue specific knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that in type 2 diabetes.

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K.3
Assays of insulin and of insulin-like activity

K.3.1
Hypoglycemic effects

K.3.1.1
Blood sugar lowering effect in rabbits

PURPOSE AND RATIONALE

A biological assay of insulin preparations in comparison with a stable standard using the blood sugar lowering effect in rabbits has been proposed already in 1925 by Harrison et al.

The biological assay of insulin using the blood sugar lowering effect in rabbits has been until recently the official assay in several pharmacopoeias, such as European Pharmacopoeia, Second Edition 1980; Deutsches Arzneibuch 1986; British Pharmacopoeia 1988; Unit-

ed States Pharmacopoeia 23 and The National Formulary 18, 1995.

The rabbit blood glucose bioassay as well as the mouse convulsion assay and the mouse glucose assay were used for establishing international standards for highly purified human, porcine and bovine insulins (Bristow et al. 1988).

In several pharmacopoeias, the biological assays have been replaced by chemical methods (British Pharmacopoeia 1999; European Pharmacopoeia, 3rd Edition 1997; but the rabbit blood sugar method is still valid in the United States Pharmacopeia USP 24, 2000.

PROCEDURE

Four groups of at least 6 randomly distributed rabbits weighing at least 1.8 kg are kept in the laboratory and maintained on a uniform diet for not less than one week before use in the assay. About 24 h before the test each rabbit is provided with an amount of food that will be consumed within 6 h. The same feeding schedule is followed before each test day. During the test all food and water is withheld until the final blood sample has been taken. The rabbits are placed into comfortable restraining cages to avoid undue excitement.

Immediately before use two solutions of the standard preparation are made, containing 1 unit and 2 units of insulin per ml, respectively, and two dilutions of the preparation being examined which, if the assumption of potency is correct, contain amounts of insulin equivalent to those in the dilutions of the standard preparation. As diluent, a solution is used of 0.1–0.25% w/v of either m-cresol or phenol and 1.4 to 1.8 w/v of glycerol being acidified with hydrochloric acid to a pH between 2.5 and 3.5.

Each of the prepared solutions is injected subcutaneously to one group of rabbits, using the same volume, which should usually be between 0.3 and 0.5 ml for each rabbit, the injections being carried out according to a randomized block design. Preferably on the following day, but in any case not more than 1 week later, each solution is administered to a second group of rabbits following a twin crossover design. One hour and 2.5 h after each injection a suitable blood sample is taken from the ear vein of each rabbit.

Blood sugar is determined by a suitable method, preferably using glucose oxidase.

EVALUATION

The results of the assay are calculated by standard analytical methods (e.g., USP 23, 1995).

CRITICAL ASSESSMENT OF THE METHOD

The classical bioassay based on blood-sugar lowering activity in rabbits has been replaced by chemical methods in some pharmacopoeias (Underhill et al. 1994), but is still included in USP 24, 2 000; and will be still necessary for evaluation of synthetic insulin derivatives.

MODIFICATIONS OF THE METHOD

An assay of insulin activity after intraperitoneal injection in **rats** has been described by Rafaelsen et al. (1965), Young(1967).

Shults et al. (1994) reported as one of the first on an implantable potentiostat-radiotelemetry system for *in vivo* sensing of glucose, implanted into the paravertebral thoracic subcutaneous tissue of a **dog**. An enzyme electrode sensor measures the oxidation current of hydrogen peroxide formed by the stoichiometric conversion of the substrate glucose and oxygen as a cofactor in an immobilized glucose oxidase layer.

Salehi et al. (1996) described the development of a compact, low power, implantable system for *in vivo* monitoring of oxygen and glucose concentrations.

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K.3.1.2 Hypoglycemic seizures in mice

PURPOSE AND RATIONALE

The biological assay of insulin using hypoglycemic seizures in mice has been suggested already in 1923 by Fraser. The biological standardization of insulin using the mouse convulsion method has been published in detail by the Health Organisation of the League of Nations in 1926 (Trevan and Boock; Hemmingsen and Krogh) and has been until recently the official assay in several pharmacopoeias, such as European Pharmacopeia, Second Edition 1980; Deutsches Arzneibuch 1986; British Pharmacopoeia 1988.

In most pharmacopoeias, the biological assays have been replaced by chemical methods (British Pharmacopoeia 1999; European Pharmacopoeia, 3rd Edition 1997).

PROCEDURE

Ninety-six mice of either sex (but not of mixed sexes) weighing 20 ±5 g are randomly distributed into 4 groups. The mice are deprived of food 2–20 h immediately preceding the test. Solutions of the insulin standard and of the test preparation containing 30 and 60 milliUnits/ml are prepared by diluting the original solution with 0.9% NaCl solution, pH 2.5. 0.5 ml/20 g mouse of these solu-tions are injected subcutaneously. The mice are kept at a uniform temperature, between 29 and 35 °C, in transparent containers within an air incubator with a transpar-ent front. The mice are observed for 1.5 h and the num-ber of mice is recorded that are dead, convulse or lie still for more than 2 or 3 s when placed on their backs.

EVALUATION

The percentage of mice of each group showing the afore mentioned symptoms is calculated and the relative potency of the test solution calculated using a 2 + 2 point assay.

CRITICAL ASSESSMENT OF THE METHOD

Attempts to replace the tests in mice and rabbits by *in vitro* tests, such as the rat diaphragm test, the rat epididymal fat pad test, or even the radioimmunoassay failed due to several reasons (Trethewey 1989). Nevertheless, for industrial production and for stability studies, the classical bioassays based on hypoglycemic seizures in mice or hypoglycemia in rabbits have been replaced by chemical methods (Stewart 1974; Underhill et al. 1994).

MODIFICATIONS OF THE METHOD

A modification of the mice seizure method using **rotating hollow cylinders** has been proposed by Young and Lewis (1947). A similar technique which increases the sensitivity of the mice seizure method has been used by Vogel (1964). The equipment consisted of seven perforated metal drums with a diameter of 15 cm. The drums were rotated in oblique position at 10 rotations per minute. Female mice weighing 16 to 20 g were deprived of feed at the afternoon before the test. On the test day, groups of 6 mice received doses of 0.5; 1.0 or 1.5 IU/kg of test preparation or standard subcutaneously and were placed after 20 min into the rotating drums. Controls received saline only. Mice with insulin-induced seizures dropped out from the rotating drum. The number of animals dropping out was counted after 15; 30; 45 and 60 min. Mice dropping out due to hypoglycemic seizures received 0.5 ml of 10% glucose solution intraperitoneally.

ED_{50} values and activity ratios were calculated according to Litchfield and Wilcoxon (1949).

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K.3.1.3 Blood sugar determinations in mice

PURPOSE AND RATIONALE

Eneroth and Ahlund (1968, 1970a,b) recommended a twin crossover method for bio-assay of insulin using blood glucose levels in mice instead of hypoglycemic seizures giving more precise results. This test was induced into the British Pharmacopoeia 1980 and continued up to 1988. Moreover, the test is included as alternative in the European Pharmacopoeia, Second Edition 1980; and in Deutsches Arzneibuch, 9. Ausgabe, 1986.

PROCEDURE

Non-fasting mice of the same strain and sex are used having body masses such that the difference between the heaviest and lightest mouse is not more than 2 g. The mice are assigned at random to four equal groups of not less than 10 animals. Two dilutions of a solution of the substance or of the preparation to be examined and 2 dilutions of the reference solution are prepared using as diluent 0.9% NaCl solution adjusted to pH 2.5 with 0.1 N hydrochloric acid and containing a suitable protein carrier. In a preliminary experiment, concentrations of 0.02 IU and 0.10 IU are tested. Each of the prepared solutions (0.1 ml/10 g body weight) is injected subcutaneously to one group of mice according to a randomized block design. Not less than 2.5 h later, each solution is administered to a second group of mice following a twin crossover design. Exactly 30 min after each injection, a sample of 50 µl of blood is taken from the orbital venous sinus of each mouse. Blood glucose concentration is determined by a suitable method, such as described by Hoffman (1937).

EVALUATION

The potency is calculated by the usual statistical methods for the twin-cross-over assay.

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K.3.2 Insulin target cells/tissues of rats[2]

K.3.2.1 Epididymal fat pad of rats

Insulin-like activity can be measured by the uptake of glucose into fat cells. Adipose tissue from the epididymal fat pad of rats has been found to be very suitable. Early studies (Beigelman 1959; Steelman et al. 1961) determined the difference of glucose concentration in the medium after incubation of pieces of epididymal rat adipose tissue or measured oxygen consumption in Warburg vessels (Ball and Merill 1961; Doisy 1963; Froesch et al. 1963). In experiments with radiolabelled ^{14}C glucose, the $^{14}CO_2$ is trapped and counted (Martin et al. 1958; Slater et al. 1961; Gliemann 1965, 1967). The principle used by Martin et al. (1958) in the first publication was measurement of $^{14}CO_2$-production from 1-^{14}C-labelled glucose by epi-didymal fat pads from the rat. This method has been used by various authors (Humbel 1959; Ditschuneit et al. 1959) and described in detail by Renold et al. (1960) and Siess et al. (1965). Sönksen et al. (1965) found a close correlation between "suppressible" insulin-like activity measured by the fad pad method and insulin concentration determined by immunoassay.

The presence of factors other than insulin in this test was proven by the persistence of serum insulin-like activity after pancreatectomy (Steinke et al. 1962).

Ball and Merrill (1961) used the manometric measurement of the net gas exchange of rat adipose tissue to quantitate small amounts of insulin.

Steinke et al. (1965) tested insulin-like activity on adipose tissue of various species, rat, mouse and guinea pig.

The epididymal fat pad assay, originally developed as bioassay of insulin-like activity in serum samples, can be used for measuring activity of synthetic insulin derivatives as well as for evaluation of peripheral insulin-like effects of compounds such as sulfonylureas. Several modifications (isolated fat cells, primary cultured adipocytes, 3T3 adipocytes) broadened the value of this assay.

A survey of the biological assays of insulin-like serum activity was given by Faulhaber and Ditschuneit (1975).

The influence of insulin on the uptake of ^{14}C glucose in cultured rabbit coronary microvessel endothelium was studied by Gerritsen and Burke (1985).

[2] Contributions by G. Müller.

K.3.2.2 Fat cells isolated by digestion with collagenase

Rodbell (1964), Gliemann (1965, 1967) used fat cells isolated by digestion with collagenase. The conversion of (3 ^{3}H) glucose to total lipids has been used as a sensitive parameter in an improved free fat cell assay for insulin-like activity by Moody et al. (1974).

Preparation of rat adipocytes

Male Wistar rats weighing 160–180 g are sacrificed and both epididymal fat pads are removed under sterile conditions. The fat pads are cut into pieces and incubated for 20 min at 37 °C with 1 mg/ml collagenase (e.g. type CLS II, Worthington, Freehold, NJ) in KRHB (Krebs-Ringer bicarbonate buffer), 25 mM HEPES/KOH (pH 7.4), 0.1 mM glucose, 1% wt/vol BSA (bovine serum albumin). The cell suspension is filtered through a 100 µm nylon screen and washed 3 times by flotation (accumulation of a thin cell layer on top of the medium after centrifugation at 1 000 *g* for 1 min in a swing-out rotor) with KRHB lacking glucose and finally suspended in the same solution. The suspension is adjusted to a final titer of 4×10^5 cells/ml.

Stimulation of rat adipocytes with insulin

Adipocytes are suspended in buffer S (Dulbecco's minimal essential medium [DME] containing 5 mM glucose, 0.5 M mM sodium pyruvate, 4 mM L-glutamine, 200 nM 1-methyl-2-phenylethyl)adenosine, 100 µg/ml gentamycine, 1% BSA and 25 mM Hepes/KOH, pH 7.4) at 5% cytocrit (corresponding to about 7×105 cells/ml). For determination of the packed cell volume, small aliquots of the cell suspension are aspirated into capillary hematocrit tubes and centrifuged for 90 s in a microhematocrit centrifuge in order to measure the fractional occupation of the suspension by the adipocytes (= cytocrit). A 20-ml portion of the adipocyte suspension (5% cytocrit) is added to 20 ml of buffer S containing human insulin as indicated. Incubations (20 min, 37 °C) are performed under 5% CO_2 in 200-ml polyethylene vials during shaking at 110 cycles/min with a stroke length of 3.5 cm.

Electroporation of rat adipocytes

For studying the molecular mechanism of insulin action and its interference by a certain compound/drug it may be useful to block known signaling or metabolic pathways in adipocytes by introduction of specific inhibitory antibodies (against key proteins/enzymes) or peptide substrates (competing for the endogenous protein substrate; e.g. synthetic peptide as kinase substrate) prior to stimulation of the adipocytes with the compounds/drugs. The antibodies and peptides can be introduced into isolated rat adipocytes by the method of electroporation without significant loss of cell viability and insulin sensitivity. 0.4 ml of buffer E (4.74 mM NaCl, 118 mM KCl, 0.38 mM $CaCl_2$, 1 mM EGTA, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mg/ml bovine serum albumin, 3 mM sodium pyruvate and 25 mM Hepes/KOH, pH 7.4) is placed in a 0.4-cm gap-width electroporation cuvette (Bio-Rad, Munich, Germany) together with the antibodies or peptides. 0.4 ml of adipocyte suspension (50% cytocrit in buffer E) is added to each cuvette and gently mixed. Electroporation is performed using a Gene Pulser Transfection Apparatus (Bio-Rad, Munich, Germany) which is set at a capacitance of 25 µF and voltage of 800 V (2 kV/cm), at 25 °C for six shocks (Shibata et al. 1991; Quon et al. 1993). After the third treatment, the adipocyte suspension is gently stirred with a plastic stick and the electric polarity is reversed. The time constant of electroporation is typically 0.6 ms during the final shock. Routinely, 4 ml of adipocyte suspension (25% cytocrit) is electroporated in five cuvettes. The time required for treatment of the five cuvettes is about 3 min. After electroporation, the cells from five electroporations are pooled and transferred to 50-ml polystyrene tubes. After incubation (30 min, 37 °C) in 5% CO_2/95% O_2, the cells are centrifuged (200 *g*, 1 min, swing-out-rotor) and the infranatant is aspirated. Thereafter the cells are washed once with 40 ml of buffer E containing 5 mM glucose and 4% BSA, suspended in 20 ml of the same buffer and then incubated (1 h, 37 °C) under 5% CO_2 prior to stimulation with insulin/compound.

MODIFICATIONS OF THE METHOD

Etherton and Chung (1981), Etherton and Walker (1982) characterized insulin sensitivity of isolated *swine* adipocytes.

K.3.2.3 Primary culture of rat adipocytes

Insulin resistant rat adipocytes can be generated *in vitro* by incubation of freshly isolated cells in primary culture in the presence of 25 mM glucose and 10 nM insulin for 20 h at 37 °C under slow shaking. When assayed for insulin-stimulated glucose transport, these cells show a right-ward shift of the dose-response curve (decreased insulin sensitivity) and a reduced maximal glucose transport velocity (decreased insulin responsiveness).

K.3.2.4 3T3 Adipocytes

Green and Kehinde (1974) isolated from an established mouse fibroblast line 3T3 two subclonal sublines that

accumulate large amounts of triglycerides when the cells are in the resting state. The cell line 3T3-L1 has been used extensively by many authors (Spooner et al. 1979; Frost and Lane 1985; Zuber et al. 1985; Chan et al. 1988; Clancy and Czech 1990; Wieland et al. 1990; Müller et al. 1993).

Kletzien et al. (1992) studied the effect of pioglitazone, an insulin-sensitizing agent, on the expression of the adipocyte fatty acid-binding protein in *ob/ob* mice and 3T3 L1 cells.

Preparation of adherent 3T3-L1 adipocytes and stimulation with insulin/compound

3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD) are seeded in 12-well (60 000 cells/well) plates and maintained in DME (high glucose) plus 10% fetal bovine serum (FBS), 5 mM L-glutamine and 2% BSA. Following three days at 100% confluence, differentiation is initiated by the addition of DME containing 10% FBS, 400 nM human insulin, 1 µM dexamethasone and 1 mM isobutylmethylxanthine. Three days later, the medium is replaced with DME plus 10% FBS and 100 nM insulin. After additional two days, the medium is changed to DME (low glucose) plus 10% FBS. Adipocytes are used 5 to 12 days after completion of the differentiation protocol, when more than 85% of the cells expressed the adipocyte phenotype. Prior to experiments, the cells are rinsed two times with low serum medium (DME containing 5 mM glucose, 0.5% BSA, 0.1% FBS, 25 mM Hepes pH 7.4, 10 mM glutamine, 100 units/ml streptomycin/penicillin), then incubated (12 to 14 h) in this medium and finally washed twice with phosphate-buffered saline [PBS] containing 2 mM sodium pyruvate prior to incubation (30 min, 37 °C) with insulin/compound.

Preparation of non-adherent 3T3-L1 adipocytes and stimulation with insulin/compound

Adherent 3T3-L1 adipocytes are serum-starved for 12 h in serum-free DME, 10 mM glutamine, 0.5% BSA, 50 units/ml streptomycin/penicillin, thereafter washed twice with PBS containing 1 mM EDTA, and then removed gently from the dishes using a rubber policeman. After washing once in PBS, the cells are maintained in suspension (30 min, 37 °C) in 4 ml of PBS containing 5 mM glucose, 1% BSA, 200 nM phenylisopropyladenosine before the addition of insulin/compound and further incubation (30 min, 37 °C).

Attachment of 3T3-L1 adipocytes to fibronectin-coated dishes

Cell culture dishes (12-well plates) are coated (4 °C, overnight) with fibronectin (10 µg/ml) or poly-L-lysine (10 µg/ml) together with vitronectin (2 µg/ml) in PBS containing 2.7 mM KCl, 6.5 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 (pH 7.4), blocked (1 h) with 0.1% BSA in PBS and then dried (1 h, 37 °C) prior to plating of the cells. Confluent 3T3-L1 adipocytes are serum-starved for 12 h in serum-free DME, 10 mM glutamine, 0.5% BSA, 50 units/ml streptomycin/penicillin and then detached by adding EDTA-trypsin (0.05 mM, 0.025%). The detached adipocytes are washed three times with PBS containing 1% BSA and then held in suspension (30 min, 37 °C) in 4 ml of buffer S prior to addition of insulin as indicated. After incubation (10 min, 37 °C), the cells are replated on dishes coated with fibronectin/polylysine in the absence or presence of 25 µg/ml peptide as indicated and further incubated (20 min, 37 °C) under 5% CO_2 (Guilherme and Czech 1998; Guilherme et al. 1998).

K.3.2.5 Lipogenesis assay

PURPOSE AND RATIONALE

This assay measures the insulin-stimulated glucose transport and conversion into lipid-soluble products (triglycerides, phospholipids, fatty acids) including the signal cascade. According to TLC-analysis of the products after enzymatic digestion, the majority of the label appears in the glycerol backbone at low glucose concentration (0.1 mM) when glucose transport is rate-limiting. At higher glucose concentrations (2 mM) when transport is not rate-limiting, two thirds of the label is incorporated into the fatty acids of the lipids.

PROCEDURE

For measurement of lipogenesis, adipocytes are incubated with D-[^{3}H]glucose (0.55 mM final concentration). The cells are lysed. The lipids separated from water-soluble products and the medium by addition of toluene-based scintillation cocktail. After phase separation, radioactivity incorporated into lipids is determined by liquid scintillation counting directly without removal of the lipid phase.

The assay is performed according to the method of Moody et al. (1974). Incorporation of D-[3-^{3}H]glucose into toluene-extractable acylglyceroides is measured after incubation of adipocytes with 1 ml Krebs-Ringer-phosphate buffer in the absence or presence of insulin or test compound.

The reaction is started by the transfer of 0.2 ml of adipocyte suspension to scintillation vials containing the following ingredients:

0.1 ml [3-^{3}H]glucose (2 µCi/ml) 0.1 mM
0.4 ml Krebs-Ringer bicarbonate solution 2-fold

0.3 ml of substance with insulin-mimetic activity or insulin

The scintillation vials are placed under a stream of carbogen for 10 s, then closed and placed in a very slowly shaking water bath (37 °C).

After incubation for 90 min, the reaction is terminated by the addition of 10 ml of toluene-based scintillation cocktail. The vials are mixed rigorously using a vortexer and subsequently left standing for 2–4 h to allow phase separation.

The ^{3}H-radioactivity is determined with a liquid scintillation counter.

EVALUATION

Blank values obtained from a typical reaction mixture containing buffer and [3-^{3}H]glucose but lacking adipocytes and substance (or insulin) have to be included in each experiment. Since the quality of the cells decreases with time (that is increase of lipid synthesis in the basal state), it is recommended to set up 2 basal incubations for every 20–25 test mixtures. The blank values (usually 500–600 dpm) are subtracted from the values measured for the corresponding set of test mixtures to correct for ^{3}H-radiation originating from the aqueous phase (i.e. [^{3}H]glucose).

Due to the very limited number of pipetting steps (the most critical one being the transfer of an equal number of cells to each assay), the standard deviations are rather low. Usually, 2–4 measurements per point are sufficient.

An insulin-dose/response curve should be performed for each experiment to test the insulin sensitivity (and thus the quality) of the cells. Appropriate insulin concentrations are 0.02, 0.04, 0.06, 0.1, 0.5, 1, 5 nM (final concentration in the assay). The maximal insulin stimulation factor for lipogenesis (at 5 nM), calculated as the ratio between [^{3}H]lipid in the presence of insulin minus [^{3}H]lipid in the absence of insulin and [^{3}H]lipid in the absence of insulin, lies in the range of 10–20-fold over the basal incorporation (absence of insulin). Half-maximal insulin stimulation is observed at 0.1–0.5 nM. The insulin-mimetic activity of substances can be expressed as % of the maximal insulin stimulation (that is as ratio between the stimulation factor for the insulin-mimetic substance and the insulin stimulation factor) to correct for the varying quality of the cells from different preparations.

MODIFICATIONS OF THE METHOD

Grunfeld and Jones (1983) explored the ability of insulin to stimulate amino acid uptake in 3T3-L1 cells using the non-metabolizable amino acid analog methylaminoisobutyric acid.

K.3.2.6 Assays of glucose transport

Total uptake of glucose

Adipocytes are incubated with D-[U-^{14}C]glucose (0.2 mM final concentration) for 20 min. Cells are separated from the medium by centrifugation on silicon oil, removed and counted for radioactivity.

This assay measures the total insulin-stimulated glucose uptake (signal cascade, glucose transport, glucose metabolism) irrespective whether the glucose is utilized via the oxidative or non-oxidative pathway. Conversion into lipids, glycogen or membrane-impermeable intermediary products (glucose-6-phosphate) will be detected. Production of lactate can not be followed.

Bypass of the insulin receptor

Adipocytes are incubated with trypsin (4 mg/ml) for 15 min at 4 °C. After addition of soy bean trypsin inhibitor (8 mg/ml) the cells are washed three times by flotation and used for determination of total uptake of glucose (see above).

This assay measures the total glucose uptake into cells with inactivated insulin receptor. Thus only compounds that bypass the first step in the insulin signal transduction cascade (binding of insulin to its receptor) will provide positive results.

Transport of 2-deoxy-glucose

The transport of 2-deoxy-D-[1-^{3}H]glucose (Amersham, specific activity 20–30 Ci/mmol, aqueous solution) by isolated rat adipocytes or 3T3 adipocytes is measured as described by Gliemann et al. (1972), Foley and Gliemann (1981), Müller and Wied (1993).

All incubations are performed in a 25 °C shaking water bath. A 50 µl adipocyte cell suspension is pipetted into minisorp tubes (Nunc, Denmark) and equilibrated at 25 °C for 30 min. Insulin (80 nM stock in KRH/5% BSA) and substances (lyophilized, 2 mg/ml) are added in 50 µl KRHB and incubated for 30 min at 25 °C. To cor-rect for 2-deoxyglucose unspecifically associated with the cell surface or entrapped in extracellular spaces, 2 µl of cytochalasin B (20 µM final conc.) from a 1 mM stock solution (in 10% ethanol, diluted with H_2O from a 10 mM stock in ethanol at the day of use) and, as a control, 2 µl of 20% ethanol were added prior to addition of radiolabeled 2-deoxy-glucose and incubated under identical conditions.

The transport assay is started by addition of 50 µl of 0.1 mM 2-deoxy-[^{3}H]glucose (2 µCi per ml of KRHB containing 10 µl/ml of ^{3}H-stock, 0.2 mCi/ml), is added and the incubation continued for another

10 (max. 20) min at 25 °C (total volume of the final incubation mixture = 150 µl).

The assay is terminated by centrifugation of 100 µl samples on top of 250 µl dinonylphthalate (Merck, Darmstadt, FRG) in 500 µl plastic tubes (Beckman) in a microfuge (10 000 *g*, 1 min, room temperature). The adipocytes remain on top of the oil, while the buffer is below the oil. The tube is cut just below the adipocyte layer, which is transferred into 5 ml aqueous scintillation cocktail (Zinsser Nr. 312 or Beckman Ready Safe) and counted.

Specific transport is calculated as the difference between total cell-associated radioactivity (absence of cytochalasin B) and unspecifically entrapped radioactivity (presence of cytochalasin B). Under the conditions used, 2-deoxyglucose transport is linear with time up to 20 min. The glucose transport stimulation factor is calculated as ratio between stimulated specific transport (presence of insulin or extract) minus basal specific transport (absence of insulin or extract) and basal specific transport. For insulin (2 nM), this factor usually lies in the range between 15 and 25.

This assay measures the insulin-stimulated specific transport of glucose across the plasma membrane via facilitated diffusion (glucose carriers) including the signal cascade irrespective of glucose utilization (as revealed by the non-metabolizable deoxyglucose). Moreover, the effect of sulfonylureas on 2-deoxyglucose transport can be demonstrated (Maloff and Lockwood 1981; Müller et al. 1994).

Clarke et al. (1984) investigated the insulin-stimulated uptake of 2-deoxy-D-glucose in cultured glial cells of rat brain.

Hernvann et al. (1987) studied the effects of human, bovine and porcine insulin on 2-deoxy-D-glucose uptake in cultured chicken embryo fibroblast monolayers.

Liu and Stock (1995) used 2-deoxy-[^{3}H]glucose uptake into various tissues for determination of tissue utilization after acute treatment with a β_3-adrenoceptor agonist.

Transport of 3-O-methylglucose

The rate of 3-*O*-methylglucose transport is determined according to Whitesell and Gliemann (1979), Karnieli et al. (1981), Basi et al. (1992) by a modification of the L-arabinose uptake method described by Foley et al. (1978).

Five ml polyethylene miniscintillation vials are prepared with 50 µl of incubation medium containing L-[1-^{3}H]glucose and 3-*O*-[methyl-^{14}C]glucose such that their concentration is 0.5 mM (58.2 and 11.6 µCi/µmol, respectively). L-Glucose is included as a marker for the extracellular space. Sugar uptake is initiated by the rapid addition of 200 µl of adipose cell suspension and then rapidly stopped by the subsequent addition of 10 µl of a 10 mM solution of cytochalasin B in 25% ethyl alcohol. The duration of this 'pulse' incubation is adjusted from 3 to 25 s so that the uptake achieved will be approximately one third of the equilibrium level. The equilibrium level of uptake is determined by incubating cells for 5 to 6 min in the presence of 3-*O*-methylglucose only and then for 1 additional min following the addition of L-glucose. Experimental blanks are obtained by adding cytochalasin B to the labeled sugar solution prior to the addition of cells. Following incubation, 200-µl samples of cells are separated from incubation medium by centrifugation through dinonylphthalate, and the separated cells and incubation medium are counted. The net uptake of 3-*O*-methylglucose is then counted, and the initial uptake velocity or rate of transport is determined.

K.3.2.7 Adipocyte plasma membranes

Preparation of rat adipocyte plasma membranes

Adipocytes are isolated from epididymal fat pads of 160–180 g male Wistar rats by collagenase digestion under sterile conditions as described above. The subcellular fractionation is performed according to McKeel and Jarett (1970), Simpson et al. (1983) with the following modifications: The postnuclear supernatant is centrifuged (12 000 *g*, 15 min). The washed pellet is suspended in 35 ml of buffer and recentrifuged (1 000 *g*, 10 min). The washed pellet (12 000 *g*, 20 min) is suspended in 5 ml of buffer, layered onto a 20 ml cushion of 38% (w/v) sucrose, 20 mM Tris/HCl (pH 7.4), 1 mM EDTA and centrifuged (110 000 *g*, 60 min, 4 °C). The membranes at the interface between the two layers (1 ml) are removed by suction, diluted with three volumes of buffer and layered on top of a 8 ml cushion of 24% Percoll, 250 mM sucrose, 20 mM Tris/HCl (pH 7.4), 1 mM EDTA. After centrifugation (45 000 *g*, 30 min) the plasma membranes are withdrawn with a Pasteur pipette from the lower fourth of the gradient (1 ml), diluted with 10 volumes of buffer and recentrifuged (150 000 *g*, 90 min, 4 °C). The pellet is suspended, recentrifuged, finally dissolved in buffer at 0.5 mg protein/ml and stored at –80 °C.

Preparation of 3T3 adipocyte homogenate and membrane fractions

Prior to homogenization, 150-mm plates are washed with DMEM as above and then once with 10 ml of PBS containing 1 mM EDTA, 0.25 M sucrose. Monolayers from four plates are scraped into 24 ml of ice-cold Tris/EDTA/sucrose buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose) and immediately homogenized with 10 strokes of a motor-driven

Teflon-in-glass homogenizer. PMSF (final concentration 0.2 mM) is added to the cell suspension prior to homogenization.

The homogenate is centrifuged (16 000 *g*, 20 min). The pellet is resuspended in 6 ml of 10 mM Tris/HCl (pH 7.4), 1 mM EDTA and applied onto a sucrose cushion (1.12 M sucrose in Tris/EDTA). After centrifugation (100 000 *g*, 1 h), the plasma membranes are removed from the top of the sucrose cushion, suspended in 25 ml of Tris/EDTA, 0.1 mM PMSF, recovered by centrifugation (30 000 *g*, 30 min) and resuspended in 0.5 ml of Tris/EDTA, 0.1 mM PMSF. The initial 16 000 *g* supernatant is centrifuged (48 000 *g*, 30 min). LDM are recovered from the supernatant by centrifugation (200 000 *g*, 2 h). The pellet is suspended in 0.5 ml of Tris/EDTA, 0.1 mM PMSF. The membrane fractions are assayed for the activity of the plasma membrane marker enzyme, 5'-nucleotidase that is enriched 7.5-fold (plasma membranes) and 0.4-fold (LDM) towards the homogenate.

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K.3.2.8 Effect on glucose transporter translocation

PURPOSE AND RATIONALE

The transport of glucose across the plasma membrane of mammalian cells represents one of the most important nutrient transport events, since glucose plays a central role in cellular homeostasis and metabolism. Apparently, rather than mediated by a single transporter molecule, glucose transport is mediated by a family of highly related transporters (Assimacopoulos-Jeannet et al. 1991; Gould and Holman 1993; Mueckler 1994):

GLUT1: the erythrocyte-type glucose transporter,
GLUT2: the liver-type glucose transporter,
GLUT3: the brain-type glucose transporter,
GLUT4: the insulin-responsive glucose transporter
GLUT5: the small intestine sugar/fructose transporter
GLUT6: a pseudogene-like sequence

GLUT4 occurs in muscle and adipose tissue. In both animal and human tissues, insulin induces a several fold increase of glucose transport. GLUT4 remains localized in intracellular vesicles in the absence of insulin. Addition of insulin recruits the transporter to the cell surface. GLUT4 is inactivated by phosphorylation in the insulin-resistant state and activated by dephosphorylation indicating an important role in the signal transduction cascade (Reusch et al. 1993).

PROCEDURE

Translocation of glucose transporters to the plasma membrane

The insulin-stimulated movement of glucose transporter molecules from intracellular stores to the plasma membrane is assayed by determination of the amount of GLUT4 protein in the plasma membrane of rat adipocytes which have been incubated for 20 min at 37 °C with insulin prior to homogenization and fractionation of the cells The isolated and purified membranes are subjected to immunoblot analysis and immunoprecipitation of GLUT4.

Immunoblot analysis of GLUT4

Polyclonal affinity-purified rabbit antibodies were raised against a synthetic peptide corresponding to the COOH-terminal domain of rat GLUT4 (residues 495–509), (James et al. 1989). The plasma membrane and LDM fractions are subjected to SDS-PAGE (25 µg protein/lane) and electro-phoretically transferred onto nitrocellulose filters (Towbin et al. 1979). Incubations of the filters with primary and secondary antibodies (^{125}I-anti-rabbit IgG from goat), autoradiography and quantitative evaluation of the immunoreactive material by densitometry are carried out as described Müller and Wied (1993).

Immunoprecipitation of GLUT4

Immunoprecipitation of GLUT4 is performed according to Müller and Wied (1993) with the following modifications: 200 µl of homogenate (100 µg of protein) is centrifuged (150 000 *g*, 60 min, 4 °C). The pellet is dissolved in 50 µl of 10 mM Tris/HCl (pH 8.0), 1 mM EDTA, 6 M urea, 5% SDS, heated (60 °C, 5 min) and again centrifuged. The supernatant is supplemented with 2.5 ml of immunoprecipitation buffer containing 1% TX-100 and then with 50 µl anti-GLUT4 antibody that has been adsorbed to protein A-Sepharose (50 µl of antiserum is incubated with 50 mg protein A-Sepharose beads in 450 µl immunoprecipitation buffer for 2 h at 4 °C under head-over rotation. The beads are centrifuged, washed two times with buffer and finally suspended in 0.5 ml of buffer). After incubation (10 h, 4 °C, head-over rotation), the immunoprecipitates are collected by centrifugation (12 000 *g*, 30 s), washed sequentially with 2 ml of immunoprecipitation buffer containing 0.5 and 0.1% TX-100 and finally dissolved in 50 µl of 10 mM Tris/HCl (pH 8.0), 1 mM EDTA, 6 M urea, 2% SDS.

The supernatant of a centrifugation is analyzed by SDS-PAGE and fluorography. The relative amount of immunoprecipitated GLUT4 is calculated from spectrophotometric analysis of the silver grains eluted from the darkened areas (bands) of the film (Suissa 1983).

The number of insulin-sensitive glucose transporter molecules in the plasma membrane is determined either indirectly by the glucose transport activity of the isolated vesicles or directly by binding of cytochalasin B (inhibitor of glucose transport that binds to the glucose transporter) to the vesicles.

Determination of cytochalasin B-binding sites

(Mühlbacher et al. 1988)

The vesicles are incubated with [4(n)-^{3}H]cytochalasin B and cytochalasin E in the presence or absence of 0.5 M D-glucose. The mixtures are rapidly spotted onto Whatman filter papers. The filters are washed, dried and counted for radioactivity. The D-glucose inhibitable cytochalasin B-binding (specific binding) is calculated as difference between total binding (– glucose) and non-specific binding (+ glucose).

Transport of glucose into plasma membrane vesicles

(Baldwin et al. 1981)

The vesicles are incubated with 0.1 mM each of D-[3-^{3}H]glucose and L-[1-^{14}C]glucose (same specific radioactivity). The incubation mixtures are spotted rapidly onto nitrocellulose filters. The filters are washed extensively, dried and counted for radioactivity. Specific transport is calculated as difference between the [^{3}H] and the [^{14}C] counts.

Both assays measure the insulin-dependent increase in total transporter number of the plasma membrane. It is generally assumed that insulin stimulates the translocation of small intracellular Golgi vesicles containing the glucose transporter molecules (low density microsomes) to the plasma membrane. Thus, the assays reflect movement to and fusion with the plasma membrane of those vesicles (including the corresponding signal transduction cascade).

MODIFICATIONS OF THE METHOD

Li end McNeill (1997) reviewed various quantitation methods for the insulin-regulatable glucose transporter (Glut4) including reconstituted glucose transport, cytochalasin B binding assays, immunocytochemistry, immunoblots, ELISA, and exofacial labels.

The effect of streptozotocin-induced diabetes on GLUT-4 phosphorylation in rat adipocytes has been studied by Begum and Draznin (1992).

The effect on glucose transporters by sulfonylureas was studied by Jacobs and Jung (1985), Jacobs et al. (1989), that of metformin by Matthei et al. (1991).

Cusin et al. (1990) found that hyperinsulinemia increases the amount of GLUT4 mRNA in white adipose tissue and decreases that of muscles.

Hofmann et al. (1991) determined GLUT4 content by Western immunoblot protein analysis and by Northern blot analysis of GLUT4 mRNA in epididymal fat

or soleus muscle tissue of streptozotocin-treated rats and of obese male KKA^Y mice after treatment with pioglitazone.

The effect of *in vivo* thyroid hormone status on insulin signaling and GLUT1 and GLUT4 glucose transport systems in rat adipocytes was studied by Matthei et al. (1995).

Galante et al. (1994) studied insulin-induced translocation of GLUT4 in skeletal muscle of insulin-resistant Zucker rats.

Recycling of GLUT4 was studied by Laurie et al. (1993), Rampal et al. (1995).

Bähr et al. (1995) studied the stimulation of myocar-dial glucose transport and glucose transporter-1 (GLUT1) and GLUT4 protein expression in cultured cardiomyocytes from rats.

Teresaki et al. (1998) studied the influence of an insulin sensitizer on GLUT4 translocation in adipocytes of rats fed a high fat diet.

Ren et al. (1995) evaluated the effect of increased GLUT4 protein expression in muscle and fat on the whole body glucose metabolism by the euglycemic hyperinsulinemic clamp technique in conscious mice.

Uphues et al. (1995) used cardiac ventricular tissue of lean and genetically obese (fa/fa) Zucker rats to study the expression, subcellular distribution and insulin-induced recruitment of the glucose transporter GLUT4.

Zeller et al. (1995) studied the GLUT1 distribution in adult rat brains using a newly developed immunoautoradiographic method.

Abe et al. (1997) reported molecular cloning of bovine GLUT4.

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K.3.2.9 Influence on further metabolic functions

Esterification

(Sooranna and Saggerson 1976)

Adipocytes are treated with low concentrations of saponin to permeabilize the plasma membrane (without disrupting internal membranes) and subsequently incubated with L-[U-^{14}C]glycerol-3-phosphate for 10 min at 25 °C. Toluene-based scintillation cocktail is added and the lipid and aqueous phases are separated by centrifugation. The lipid phase is removed and counted for radioactivity.

This assay measures the insulin-stimulated esterification of glycerol-3-phosphate into lipid-soluble products (triglycerides, phospholipids) including the signal cascade. Glucose transport does not interfere as revealed by lack of inhibition of basal esterification by cytochalasin B.

Glycerol-3-phosphate-acyltransferase (GPAT)

Vila et al. (1990) showed that insulin activates glycerol-3-phosphate-acyltransferase through a phospholipid-derived mediator.

Glycerol-3-phosphate-acyltransferase is assayed as follows: The incubation mixture contains 50 mM Tris/HCl (pH 7.4), 200 mM KCl, 1 mM DTT, 150 µM palmitoyl-CoA, 2 mg/ml BSA, 0.2 mM [^{3}H]glycerol-3-phosphate (2 µCi) and homogenate from 3T3 adipocytes in a final volume of 0.5 ml. The reaction is started by addition of homogenate. After incubation (3 min, 37 °C) the reaction is stopped with 2 ml of water-saturated butanol, followed by 1.5 ml of butanol-saturated water. The butanol phase is separated and washed twice. An aliquot is counted for radioactivity. Under these conditions the rate of product formation is linear for at least 15 min. The radiolabeled products formed are primarily phosphatidic acid (65–70%) and lyso-phosphatidic acid (20–25%) as determined by thin layer chromatography.

Time- and concentration dependence of sulfonylurea-stimulated glycerol-3-phosphate-acyltransferase was tested by Müller et al. (1994).

Measurement of lipolysis as release of NBD-FA

For fluorescent labeling of adipocyte lipids, 200-µl portions of adipocyte suspension in 1.5-ml plastic cups are incubated with 200 µl of KRH containing 0.75% BSA and 1 mM glucose in the presence of 50 µl insulin (5.5 nM or as indicated) for 15 min at 37 °C prior to addition of 50 µl of 12-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) dodecanoic acid (NBD-FA; 500 µM, prepared daily by dissolving 39.8 mg NBD-FA in 1 ml ethanol under moderate heating and subsequent 1 : 200 dilution with KRH containing 0.75% BSA) and further incubation for 60 min at 37 °C under mild shaking (thermomixer 5 436, setting 10, Eppendorf, Hamburg, Germany). For removal of the free NBD-FA left in the incubation medium, the adipocyte suspensions are combined in 50-ml plastic vials and centrifuged (500 *g*, 1 min, swing-out rotor). The infranatant below the cell layer is aspirated. The adipocytes are suspended in 30 ml of Medium 199 (HEPES modification) containing 4 mM L-glutamine, 10 mg/ml glucose, 30 mg/ml BSA. After two further cycles of centrifugation and resuspension, the washed adipocytes are finally adjusted to 5% lipocrit with Medium 199. For initiation of lipolysis, 400-µl portions of adipocyte suspension are supplemented with 500 µl of Medium 199 containing glutamine, glucose and BSA as indicated above and incubated in a total volume of 1 ml in the presence or absence of isoproterenol, adenosine deaminase or insulin and for periods of time indicated in plastic scintillation vials at 37 °C under mild shaking in a water bath. Methods I and II: The samples are transferred into pre-cooled Eppendorf cups containing 500 µl chloroform, mixed vigorously and centrifuged (12 000 *g*, 2 min). Aliquots of the lower phase is removed and 5- or 10-µl portions analyzed by TLC

using Si-60 plates and diethylether/petrolether/acetic acid (78 : 22 : 1, v/v) as solvent system. Quantitative evaluation of the amount of NBD-FA released is performed by fluorescent imaging (Molecular Dynamics, Storm 840) using imaging software (Molecular Dynamics, Program Image Quant) (Method I). Alternatively, 200-µl aliquots of the lower phase are supplemented with 3.25 ml of methanol/chloroform/heptane (10 : 9 : 7, v/v) and 1.05 ml of 0.1 M potassium carbonate, 0.1 M boric acid (pH 10.5), vortexed and centrifuged (800 *g*, 20 min). Aliquots of the upper phase (1.5 ml) are removed and measured for fluorescence in an automatic fluorometric analyzer (Cobas Bio, Roche Diagnostic, Welwyn, UK) (Method II). Method III: 300-µl aliquots of the samples are filtered under vacuum over GF/C-glass microfiber filters (Whatman, Maidstone, UK) that had been pre-wetted with butanol-saturated water. The filters are washed once with 300 µl butanol-saturated water and once with 300 µl water-saturated butanol. After centrifugation, portions of the water phase are used for glycerol determination (see below).

Measurement of lipolysis as release of glycerol or generation of cAMP

400 µl of adipocyte suspension (lipocrit 10%) are suspended in 600 µl KRH containing 0.75% BSA, 1.5 mM glucose, and incubated in the absence or presence of isoproterenol, adenosine deaminase or insulin at 37 °C in a mildly shaking water bath. 200-µl aliquots are transferred into Eppendorf cups containing 20 µl $HClO_4$ (70%), vortexed vigorously and centrifuged (12 000 *g*, 15 min, room temperature). 110 µl of the infranatant is removed, taking care not to aspirate any of the precipitate, supplemented with 60 µl of 1 M Tris/HCl (pH 7.5), incubated for 15 min on ice and then centrifuged (13 000 *g*, 15 min, 4 °C). 150 µl of the infranatant is removed for glycerol determination, frozen in liquid N_2 and stored at –20 °C prior to determination of glycerol and cAMP levels. Glycerol is measured fluorometrically by a modified version of the enzymatic method by Wieland (Wieland 1974). The reaction mixture contained 0.1 M Tris/HCl (pH 9.3), 0.9 mM ATP, 1.25 mM NAD, 0.1 M $MgCl_2$, 0.25% (v/v) hydrazine hydrate, 5–50 µl sample or standard, and a mixture of glycerokinase (0.2 U/ml final conc.) and glycerol 3-phosphate dehydrogenase (0.7 U/ml final conc.) in a total volume of 240 µl. Fluorescence is determined using an automatic fluorometric analyzer. cAMP is determined by a modification of the protein kinase binding procedure. Samples and standards are incubated with 8 µg of R-subunit of protein kinase A and 175 nCi [8-^{3}H]cAMP in a buffer composed of 50 mM Tris/HCl (pH 7.4), 4 mM EDTA in a final volume of 200 µl for 4 h at 4 °C to reach equilibrium. Charcoal suspension (30 g/l) is added and after vortexing, the mixture is centrifuged at 16 000 *g*, 2 min, 4 °C). The supernatant is transferred into scintillation vials, supplemented with 5 ml water-compatible scintillation fluid and counted for radioactivity using a liquid scintillation counter. The concentration of endogenous unlabelled cAMP in the samples is determined from a linear standard curve.

Measurement of lipolysis as PKA activity ratio

500-µl samples are withdrawn from the fluorescent labeled adipocytes, transferred to 2-ml microfuge tubes, pre-cooled to 4 °C, containing at final concentrations: 20 mM Tris/HCl (pH 7.4), 10 mM EDTA and the cAMP phosphodiesterase inhibitor, Ro 20-1 724 (100 µM). The mixture is vortexed briefly, decanted into a 5-ml Teflon-in-glass homogenizer and homogenized with 10 strokes of the rotating (1 500 rpm) Teflon pestle. The homogenate is transferred to pre-cooled microfuge tubes and centrifuged (16 000 *g*, 10 min, 4 °C). The infranatant is removed and immediately assayed for PKA activity. 25 µl of assay medium (20 mM MOPS/KOH, pH 7.0, 10 mM $MgCl_2$, 0.1 mM ATP, 0.5 mg/ml histone H1, 5 mM DTT, 50 µCi/ml [γ-^{32}P]ATP) is added to 25 µl infranatant. After vortexing and incubation for 20 min at 30 °C, the reaction is terminated by addition of 1 ml of ice-cold 20% TCA containing 5 mM sodium pyrophosphate and incubation (1 h, 4 °C). The precipitates are sedimented by centrifugation (16 000 *g*, 5 min, 4 °C) and, after removal of the supernatant, dissolved in 100 µl of cold 1 N NaOH and re-precipitated with 20% TCA and 5 mM sodium pyrophosphate. The precipitate is then filtered under vacuum over GF/C-glass microfiber filter that had been pre-wetted with 5% TCA, 5 mM sodium pyrophosphate. The filters are washed with 4 ml of the same medium, dried and counted for radioactivity. Each adipocyte infranatant is assayed in quadruplicate under four separate conditions: (I) with no further additions; (II) in the presence of 15 µM cAMP; (III) in the presence of 2 µM A-kinase inhibitor synthetic peptide and (IV) in the presence of 15 µM cAMP and 2 µM A-kinase inhibitor synthetic peptide. The results are expressed as corrected PKA activity ratios (I)–(III)/(II)–(IV) (Honnor et al. 1985a; Honnor et al. 1985b; Londos et al. 1985).

Protein phosphatase 2A (PP2A) activity

Insulin inhibits lipolysis by decreasing the phosphorylation and thus the activation state of hormone-sensitive lipase in adipose tissue via both inhibition of PKA and stimulation of protein phosphatase 2A (PP2A) by at present unknown mechanisms. For measurement of PP2A activity, the adipocytes (3×10^7 cells) are washed once with 25 ml of ice-cold 100 mM Tris/HCl (pH 8.5), 10 mM EDTA, 25 mM DTT and immediately resuspended in 0.5 ml of 100 mM Tris/HCl (pH 7.0), 2 mM

EDTA, 10 mM DTT, 0.5 mM benzamidine, 0.2 mM PMSF, 2 µg/ml leupeptin, 5 µg/ml pepstatin. After addition of the same volume of ice-cold glass beads, cell extracts are prepared by vigorous vortexing (5 times 5 s each with cooling intervals on ice) and centrifuged (13 000 *g*, 5 min, 4 °C). The supernatant (S13) is diluted with 3 volumes of the same buffer containing 1% BSA. Protein phosphatase activity is assayed as the ability to dephosphorylate ^{32}P-labeled myelin basic protein. 10 µl of S13 is added to an assay mixture (total volume 50 µl) containing 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.5 mM EGTA, and 0.2 nmol ^{32}P-labeled myelin basic protein (900 dpm/pmol) for 20 min at 30 °C in the absence or presence of 2 nM okadaic acid. The reaction is terminated by the addition of 50 µl of ice-cold 10% TCA. After incubation for 15 min on ice and centrifugation (12 000 *g*, 5 min, 4 °C), the supernatant is neutralized with NaOH and measured for radioactivity by liquid scintillation counting (10 ml Aquasol). PP2A activity is determined as the difference between ^{32}P-radiolabel measured in the presence of okadaic acid (10 nM) corresponding to the PP2A independent phosphatase portion and the label determined after uninhibited incubation corresponding to the total phosphatase activity. Under these conditions, release of $^{32}P_i$ is linear for 10 min. One unit of activity is defined as the amount of enzyme that catalyzes the release of 1 µmol of $^{32}P_i$-radiolabel from labeled myelin basic protein in one min under conditions of the standard assay (Müller et al. 2000).

cAMP-dependent protein kinase (PKA)

Insulin inhibits PKA by an at present unknown mechanism.

cAMP-dependent protein kinase is assayed according to Roskoski (1983, Honnor et al. 1985): The reaction mixture contains 25 µl of rat adipocyte cytosol, 0.4 µM kemptide, 40 mM Tris/HCl (pH 7.2), 2 mM DTT, 12.5 mM $MgCl_2$, 0.1 mM PMSF, 1 mM IBMX, 100 µM [γ-^{32}P]ATP (1 µCi) with or without 1 µM cAMP in a total volume of 100 µl. After incubation (10 min, 30 °C) the reaction mixture is chilled on ice, then supplemented with 3 ml of 75 mM phosphoric acid, 100 mM NaF, 10 mM ATP and immediately spotted on phosphocellulose filters (Whatman P18). After extensive washing with 75 mM phosphoric acid, the filters are dried and counted for radioactivity. The PKA activity is expressed as the ratio between ^{32}P-incorporation into kemptide without and with cAMP. This activity ratio is a parameter for the portion of PKA active *in vivo* toward total cellular PKA at the time point of homogenization.

The inhibition of isoproterenol-stimulated proteinkinase A by sulfonylureas was tested by Müller et al. (1995).

The effect of tolbutamide and glyburide on cAMP-dependent protein kinase activity in rat liver cytosol was investigated by Okuno et al. (1988).

Insulin-sensitive cAMP-specific phosphodiesterase (PDE)

Membrane-bound phosphodiesterase of rat epididymal fat cells is stimulated when intact cells are exposed to insulin. The localization and properties of the insulin-sensitive cAMP phosphodiesterase have been described by Kono et al. (1975), Osegawa et al. (1982), Saltiel and Steigerwalt (1985), Solomon et al. (1986).

The low density microsomal fraction of 3T3 adipocytes is incubated with insulin or test compounds at various concentrations for 20 min. For the assay of cAMP-specific PDE up to 50 µl of low density microsomal fraction (LDM) from rat adipocytes are incubated (5 min, 30 °C) with 500 nM [2,8-^{3}H]cAMP (100 nCi) in 50 mM Tris/HCl (pH 7.4), 0.5 mM DTT, 5 mM $MgCl_2$, 50 µM PMSF in a total volume of 0.25 ml. The incubation is terminated by the sequential addition of 30 µl of 10 mM IBMX and 120 µl of 0.1 N HCl and heating (5 min, 95 °C). After neutralization (120 µl of 0.1 N KOH, 80 ml of 250 mM Tris/HCl pH 7.4), 10 µl of crude 5'-nucleotidase (5 mg/ml) (*Crotalus atrox*) is added to the mixture. The reaction (30 min, 37 °C) is terminated by the addition of 60 µl of 200 mM EDTA, 5 mM adenosine. Unreacted cAMP is removed by the addition of 1 ml of a 1 : 3 slurry of Dowex AG-1X8 (Biorad, München, FRG). Solutions are shaken (5 min, 4 °C) and centrifuged (1 000 *g*, 5 min). Radiolabeled adenosine left in the supernatant is determined by liquid scintillation counting. The assay is proportional up to 100 µg of protein and linear throughout 20 min.

Calculation of insulin-mimetic activity

Data from metabolic assays are calculated as stimulation factor above basal activity (absence of insulin/compound) (e.g. for lipogenesis, glycerol-3-phosphate acyltransferase, glucose transport and GLUT4 translocation) or as difference between the basal and insulin/compound-induced values for inhibition of lipolysis or vice versa for stimulation of glycogen synthase and in each case normalized to the basal (set at 0%) and maximal insulin action (set at 100%; elicited at 10 nM insulin). At least three different cell preparations with two to four independent incubations with insulin/compound for each preparation should be performed. Each incubation should be assayed at least in triplicate. Concentration/response curves can be fitted to the equation

$$y = a + b\,[x / (x + k)]$$

using a Marquardt-Levenberg nonlinear least squares algorithm. When plotted on linear-log axes, this equa-

tion gives a sigmoidal curve where the parameters are associated with the following properties:

a = basal response;
$a + b$ = maximal response;
k = half-maximal concentration (EC_{50});
x = concentration of insulin.

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K.3.2.10 Insulin signaling cascade

PURPOSE AND RATIONALE

Bipartite metabolic-mitogenic signaling by insulin

Upon binding of insulin to the insulin receptor in insulin-responsive cells (adipocytes, myocytes, hepatocytes), the insulin signal transduction machinery, a complex network of protein-protein interactions and protein (serine/threonine/tyrosine) phosphorylation cascades between a multitude of cellular signaling components, is activated and transmits the insulin signal to a variety of metabolic (predominantly) and mitogenic (to a minor degree) effector systems (e.g. Glut4 translocation, gene expression) (Myers and White 1995; Saltiel 1996; Yenush and White 1997; White 1998). For compounds/drugs with insulin-mimetic/sensitizing activity, it is important to differentiate between metabolic and mitogenic signaling in insulin-responsive cells, such as adipocytes.

Two pathways within the insulin signaling pathway have been dissected and are thought to mediate the different biological functions of the hormone: (I) Activation of phosphatidylinositol-3'-kinase (PI 3-K) plays a pivotal role for regulation of glucose transport and cellular trafficking as well as glycogen synthesis and lipolysis by insulin. (II) Formation of the Shc-Grb2 complex leads to activation of the Ras-pathway which has been linked to insulin regulation of both cell growth and gene expression, although this has been questioned recently. Many of the proteins involved in these two pathways have been identified at the molecular level (White 1996; White 1997; Holman and Kasuga 1997). The insulin receptor is a transmembrane tyrosine kinase which when activated by insulin binding, undergoes rapid autophosphorylation and phosphorylates a number of intracellular substrates, among them one or more 50- to 60-kDa proteins including Shc, a 15-kDa fatty acid binding protein and several so-called insulin receptor substrate proteins, IRS-1/2/3/4. Following tyrosine phosphorylation, the IRS polypeptides act as docking proteins for several Src homology 2 domain-containing adaptor molecules and enzymes, including PI 3-K, Grb2, SHP2, Nck, and Fyn. The interaction between the IRS proteins and PI 3-K occurs through the p85 regulatory subunit of the enzyme and results in an increase in catalytic activity of the p110 subunit. PI 3-K is essential for many insulin-sensitive metabolic processes including stimulation of glucose transport and glycogen synthesis. In all cases in which there is stimulation of tyrosine phosphorylation of IRS proteins, there is concomitant docking of these proteins to the p85 subunit of PI 3-K and this

docking is associated with stimulation of the PI 3-K activity.

It is generally accepted that one branch of acute metabolic insulin signalling is initiated by activation of PI 3-K (Yeh el al 1997; Alessi and Downes 1998; Shepherd et al. 1998). This may be necessary for insulin stimulation of GS and glucose transport and even be sufficient for the latter (Cheatham et al. 1994; Herbst et al. 1995; Yeh et al. 1997). Further, there is experimental evidence for protein kinase B (PKB) being an element of the signalling cascade leading from PI 3-K to activation of glycogen synthase (GS) and glucose transport in isolated rat adipocytes, various cell culture models and human skeletal muscle (Burgering and Coffer 1995; Hurel et al. 1997; Shepherd et al. 1997). The experimental data available favor a model for a cascade which assumes the following sequence of events (Gustafson et al. 1998): PI 3-K activation results in the production of phosphatidylinositol (3,4,5)-trisphosphate and phosphatidylinositol (3,4)-bisphosphate. The binding of the pleckstrin-homology domain of PKB to these phosphoinositides both recruits PKB to the plasma membrane and directly and/or indirectly (via phosphorylation by the membrane-associated serine/threonine protein kinases, PDK1/2) stimulates its kinase activity. Activated PKB phosphorylates substrate proteins (e.g. GSK-3 which is thereby inhibited, leading to dephosphorylation and activation of GS), resulting in a variety of biological effects possibly including stimulation of glycogen synthesis and GLUT4 translocation. Thus the current knowledge favors the view that most metabolic insulin signals emerge from insulin-dependent tyrosine phosphorylation of the IRS proteins whereas the mitogenic insulin action is apparently coupled to tyrosine phosphorylation of the Shc proteins. These serve as docking sites for the Grb-SOS complex which possesses GDP-GTP exchange activity for the small G-protein Ras and is activated by binding to Shc. GTP-loaded Ras interacts with and activates the Raf serine kinase which phosphorylates the dual specificity kinase mitogen/extracellular signal-activated kinase MEK (= MAPKK). Activated MEK phosphorylates the mitogen-activated protein kinases MAPK, ERK1 (p44) and ERK2 (p42), which in turn phosphorylate and activate a number of transcription factors (e.g. c-jun, c-fos) ultimately leading to increased gene and protein expression as well as DNA synthesis in insulin-like fashion (Gustafson et al. 1998).

In addition to the identification of the signal transduction pathways directly leading from the insulin receptor to downstream targets, several cross talks have been delineated between signalling transmission by insulin and other hormones/growth factors or diverse exogenous stimuli which either mimic (to a certain degree) or modulate in a positive or negative fashion metabolic and/or mitogenic insulin action in various cellular systems. Since none of these ligands activates the insulin receptor kinase directly, their signalling pathways may converge with that of insulin at a more distal signalling step (Argetsinger et al. 1995; Huppertz et al. 1996; Kowalski-Chauvel et al. 1996; Velloso et al. 1996; Ricort et al. 1997; Verdier et al. 1997; Baron et al. 1998). Soluble phosphoinositolglycan molecules which have been shown to exert partial insulin-mimetic effects in diverse cellular and subcellular systems (Müller et al. 1997; Kessler et al. 1998; Frick et al. 1998) can also be classified into the latter category. Interestingly, the second-generation sulfonylureas, glibenclamide and, in particular, glimepiride have been demonstrated to stimulate glucose transport and non-oxidative metabolism in adipose and muscle cells *in vitro* by causing insulin receptor-independent tyrosine phosphorylation of IRS-1/2 and stimulating the downstream located insulin signaling events (Müller et al. 1994; Müller and Geisen 1996). This insulin-mimetic signaling in cells of peripheral tissues may explain the insulin-independent blood glucose-lowering activity of glimepiride/glibenclamide as has been reported in a number of animal studies (Geisen 1988; Müller et al. 1995), which supplements the potent blood glucose decrease via insulin release provoked by these anti-diabetics.

PROCEDURES USING CULTURED ADIPOCYTES

Preparation of cell lysates

Stimulated adherent or non-adherent 3T3-L1 cells are placed on ice and then washed twice with 50 mM HEPES/KOH, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM EDTA, 10 mM glycerol-3-phosphate, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 100 mM NaF (in case of non-adherent cells by flotation [200 *g*, 2 min] and aspiration of the infranatant). Cells are solubilized in the above buffer containing 1% (vol/vol) Triton X-100, 0.1% sodium deoxycholate, 10% glycerol and protease inhibitors (20 µg/ml leupeptin, 10 µg/ml pepstatin A, 50 µg/ml aprotinin, 10 µM E-64, 0.5 mM phenylmethylsulfonylfluoride (PMSF) = lysis buffer) for 30 min on ice (non-adherent cells) or by scraping with a Teflon policeman (adherent cells). Total lysates are centrifuged (25 000 *g*, 20 min, 18 °C). The infranatant is aspirated taking care to avoid contamination by the upper fat layer and re-centrifuged to obtain the defatted cell lysate. Stimulated rat adipocytes are washed once with PBS containing 2 mM sodium pyruvate by flotation (200 *g*, 2 min) and aspiration of the infranatant and immediately homogenized in lysis buffer containing 4 mM benzamidine by 10 strokes/200 rpm in a medium-fitting Teflon-in-glass homogenisator (2 ml vessel vol.) at 18 °C. Defatted cell lysate is prepared as described above.

Immunoprecipitation

Defatted cell lysates (standardized for 5 to 10 µg protein) are precleared (1 h, 4 °C) with protein G/A-Sepharose and then supplemented with appropriate antibodies (often 0.5–5 µg/sample) preadsorbed on protein G-Sepharose (monoclonal antibodies) or protein A-Sepharose (rabbit antibodies) in a total volume of 100 µl of lysis buffer. After incubation (4 h, 4 °C, end-over-end rotation) and centrifugation (13 000 *g*, 2 min, 4 °C), the collected immune complexes are washed twice with 1 ml each of immunoprecipitation buffer (50 mM HEPES/KOH, pH 7.4, 500 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4) containing 1% NP-40 (omitted for sequential immunoprecipitation), then twice with 1 ml each of immunoprecipitation buffer containing 150 mM NaCl and 0.1% NP-40 and once with 1 ml of immunoprecipitation buffer lacking salt and detergent and finally suspended in 50 µl of Laemmli buffer (2% SDS, 5% 2-mercaptoethanol), heated (95 °C, 2 min) and centrifuged. The supernatant samples are analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE; 4–12% Bis-Tris precast gel, pH 6.4, morpholinoethanesulfonic acid (MES)/SDS running buffer, Novex, San Diego, Ca) under reducing conditions. For sequential immunoprecipitation, the supernatant samples (50 µl) are supplemented with 1 ml of immunoprecipitation buffer containing 1% NP-40 and 1–10 µl of the relevant antiserum. After incubation (12 h, 4 °C), 50 µl of protein A-Sepharose (100 mg/ml in immunoprecipitation buffer) is added and the incubation continued (4 h, end-over-end rotation). The immune complexes are collected, washed and processed for SDS-PAGE as described above.

Immunoblotting

Proteins are transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, Eschborn, Germany). The blocked membrane is incubated (2 h, 25 °C) with the appropriate antibodies (often 1 : 200–1 : 5 000; or e.g. against phosphotyrosine [2 µg/ml]), washed (four times with Tris-buffered saline [TBS] containing 1% (vol/vol) Nonidet-P40 [NP-40] and 0.5% Tween 20, twice with TBS containing 0.5% Tween 20, twice with TBS) and then incubated (1 h, 25 °C) with appropriate horseradish peroxidase-coupled detection (anti-mouse or rabbit) antibodies (1 : 15 000 dilution; enhanced luminescence, Pierce, Rockford, IL, USA) in TBS containing 5% (wt/vol) BSA, washed (five times with TBS containing 1% NP-40 and 0.5% Tween 20, three times with TBS containing 0.05% Tween 20 and finally processed with chemiluminescent reagents (Renaissance Chemiluminescence Detection System, NEN/DuPont, Bad Homburg, Germany) and subjected to phosphorimaging.

Immune complex kinase assays

The kinase immune complexes are washed in kinase buffer (50 mM Hepes/KOH, pH 7.4, 100 mM NaCl, 5 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM DTT, 1 mM Na_3VO_4) and then suspended in 50 µl of kinase buffer. The kinase reactions are started by addition of ATP (unlabeled or ^{32}P-labeled; final conc. often 40 µM, 0.2 mCi/ml – 100 µM, 0.5 mCi/ml) and incubated (often 3–15 min; 22 °C) in the absence (autophosphorylation) or presence of recombinant substrate protein (often 0.1–1 µg). Autophosphorylation reactions are terminated by addition of 50 µl of ice-cold stop buffer (50 mM Hepes/KOH, pH 7.4, 150 mM NaCl, 100 mM ATP, 0.05% Triton X-100) and washing of the beads twice with 1 ml of stop buffer prior to addition of 20 µl of Laemmli buffer and boiling (95 °C, 5 min). Substrate phosphorylation reactions are terminated by addition of 10 µl of four-fold concentrated Laemmli buffer and boiling. The phosphoproteins are separated on SDS-PAGE (10% Bis-Tris resolving gel, morpholinopropanesulfonic acid/SDS running buffer) and analyzed by phosphorimaging directly (use of [^{32}P]ATP) or after immunoblotting with anti-phosphotyrosine antibody (use of unlabeled ATP). Under these conditions the kinase reactions are linear with time for the assay period. Protein concentration is determined by the bicinchoninic acid protein assay protocol from Pierce (Rockford, IL, USA) with crystalline BSA as standard. Phosphorimaging is performed with a phosphorimager Storm 860 and quantitatively evaluated using ImageQuant software (Molecular Dynamics). Differences in recovery in the amounts of immunoprecipitated protein during a specific experiment are corrected in each case (data on fold- or % stimulation) for the amount of protein actually applied onto the gel by homologous immunoblotting. The data should be confirmed by running independent experiments with different batches of adipocytes several times each with two to five parallel independent immunoprecipitation/kinase assay/immunoblotting analyses.

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K.3.2.11 Insulin signaling in the liver

PURPOSE AND RATIONALE

In NIDDM patients with moderate fasting hyperglycemia, the liver production of glucose is increased by about 0.5 mg/kg/min, or about 50 g/d above normal (DeFronzo et al. 1992). This modest increase is the consequence of reduced suppression of hepatic glucose production by insulin (Firth et al. 1987) and could be nullified by reducing dietary carbohydrate by 50 g/d. The increased hepatic glucose production in NIDDM is probably caused by a combination of lack of an insulin-mediated reduction in glucagon secretion (Müller et al. 1970) and hepatic resistance to insulin action at the level of both the insulin signaling transduction cascade and its coupling to the metabolic end effector enzymes of gluconeogenesis. The importance of increased hepatic glucose production is underlined by the fact that when phosphoenolpyruvate carboxykinase (PEPCK), the key rate-limiting regulatory enzyme of gluconeogenesis, is overexpressed in mice carrying a transgene expressing the protein hyperglycemia results (Valera et al. 1994). Independent of whether increased

hepatic glucose production plays a primary or only secondary role in the pathogenesis of human NIDDM, analysis of insulin signaling and action in liver is important for gaining a complete picture of the pathophysiology of NIDDM. The model of the isolated perfused rat liver offers advantages determining insulin action, which, compared to primary and cultured liver cells, are the intact tissue, the near physiological function of the liver, and, compared to the *in vivo* situation, the separation from other effects which may also affect hepatic metabolism, like basal glucagon secretion from β-cells, increased sympathetic activity and/or hypothalamic effects (Nonogaki 2000).

Preparation of rat liver cytosol

Portions of frozen liver (0.2–1 g wet weight), which has been perfused as described in K.6.2.1., are homogenized in a buffer containing 50 mM Hepes/KOH (pH 7.4), 140 mM NaCl, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM EDTA, 2.5 mM Na_3VO_4, 10 mM glycerol-3-phosphate, 20 mM $NaPP_i$, 20 mM NaF, 1 mM phenylphosphate, 5 µM okadaic acid (sodium salt), 1% Nonidet P-40, 10% glycerol and protease inhibitors (10 µg/ml leupeptin, 5 µg/ml pepstatin A, 75 µg/ml aprotinin, 100 µM benzamidine, 2 µg/ml antipain, 10 µg/ml soybean trypsin inhibitor, 5 µM microcystin, 5 µM E-64, 0.2 mM PMSF) using an Ultraturrax T25 basic (three 10-s cycles at 2 000 rpm on ice) and then a tight-fitting Teflon-in-glass homogenizer (5 strokes at 500 rpm on ice). The total homogenate was centrifuged (30 min, 48 000 *g*, 4 °C). The supernatant was carefully removed to avoid contamination with the upper fat layer and re-centrifuged. The fat-free supernatant obtained was stored in liquid N_2 and used as cytosol (3–5 mg protein per ml) for immunoprecipitations and PEPCK activity measurements.

Immunoprecipitation of IRß, IRS-1/2 and GSK3

Up to 1 ml portions of cytosol (equal amounts of protein) are precleared (30 min, 4 °C) with protein A-Sepharose (50 mg, Pharmacia) and then supplemented with appropriate antibodies (anti-IRS-1 [rabbit, polyclonal, protein A-purified, raised against a peptide corresponding to amino acids 1 220–1 235 of rat IRS-1, Upstate Biotechnology]: 3 µg/ml; anti-IRS-2 [raised against peptide mixture corresponding to amino acids 618–747 and 976–1 094 of mouse IRS-2]: 1 : 100; anti-IRß [raised against a peptide corresponding to the 100 carboxy-terminal amino acids of human IR, Upstate Biotechnology]: 5 µg/ml; anti-GSK3ß [mouse monoclonal, protein G-purified, raised against a peptide corresponding to amino acids 203–219 of Drosophila GSK3, Upstate Biotechnology, Lake Placid, NY]: 2.5 µg/ml, preadsorbed on protein A-Sepharose. After incubation (4 h, 4 °C, end-over-end rotation) and centrifugation (3 000 *g*, 2 min, 4 °C), the collected immune complexes are washed twice with 1 ml each of immunoprecipitation buffer (50 mM Hepes/KOH, pH 7.4, 500 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM $NaPP_i$, 2.5 mM Na_3VO_4) containing 1% Nonidet P-40, then twice with 1 ml each of immunoprecipitation buffer containing 150 mM NaCl and 0.2% Nonidet P-40 and once with 1 ml of immunoprecipitation buffer lacking salt and detergent and finally suspended in 50 µl of Laemmli buffer, heated (95 °C, 2 min) and centrifuged. The supernatant samples are analyzed by SDS-PAGE (4–12% Bis-Tris Novex precast gel, pH 6.4, morpholinoethanesulfonic acid/SDS running buffer) under reducing conditions.

Immunoblotting

Immunoblotting is performed as described previously (Frick et al. 1998; Müller et al. 2000) with minor modifications. Briefly, after SDS-PAGE and transfer of the proteins to polyvinylidene difluoride membranes (2 h, 400 mA in 20% methanol, 192 mM glycine, 25 mM Tris, 0.005% SDS), the blocked membrane (1 h in blotting buffer containing 20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20, 0.1% Brij, 0.01% NP-40 and with 1% ovalbumin and 1% BSA (anti-phosphotyrosine, anti-IRß, anti-GSK-3) or with 5% non-fat dried milk (anti-p85, anti-IRS-1/2) was incubated (2 h, 25 °C) with anti-IRß (3 µg/ml), anti-IRS-1 (rabbit polyclonal, immunoaffinity-purified, raised against a peptide corresponding to the 14 carboxyterminal amino acids of rat IRS-1, 1 : 500), anti-IRS-2 (1 : 250), anti-p85α (rabbit, polyclonal, protein A-purified, raised against full-length rat p85 PI-3K, Upstate Biotechnology, 1 µg/ml), anti-GSK3 (phosphoserine 21- and 9-specific; New England Biolabs 9 331, 1 : 250) or anti-phosphotyrosine (1 : 1 000, Transduction Laboratories, pY20) antibodies, and then washed five times with blotting buffer containing the corresponding blocking reagent. After incubation (1 h, 25 °C) of the membranes [^{125}I]protein A (5 µCi/ml, Amersham-Buchler) in the same blocking medium, the membranes were washed five times and then subjected to autoradiography (Kodak X-Omat AR) or phosphorimaging (Molecular Dynamics, Storm 860). Quantitative analysis of the blots was performed by using IMAGEQUANT software. The recovery in the amounts of immunoprecipitated protein was corrected (data on fold- or % stimulation) for the amount of protein actually applied onto the gel as revealed by homologous immunoblotting. Each experiment was performed with samples from four different liver perfusions (as indicated in the figure legends) with three to five independent immunoprecipitation/kinase assay/immunoblotting procedures.

Determination of PI-3K activity

IRS immunecomplexes are incubated (10 min, 22 °C) in 50 µl of 20 mM Tris/HCl (pH 7.0), 50 µM [γ-^{33}P]ATP (5 µCi, New England Nuclear), 10 mM $MgCl_2$, 2 mM MnCl, 100 mM NaCl, 2 mM EDTA, 0.5 µM wortmannin (for control incubations, only) containing 10 µg of phosphatidylinositol (PI, Avanti Polar Lipids, Alabaster, AL) and 1 µg of phosphatidylserine (Folli et al. 1993). After addition of 10 µl of 8 M HCl and 160 µl of a 1 : 1 mixture of methanol/chloroform, the extracted phospholipids are resolved by TLC on plates coated with 1% oxalate and developed in chloroform:methanol: water:ammonia (60 : 47 : 11.3 : 3.2, v/v). Radiolabeled phosphatidylinositol 3-phosphate (PI 3-P) is visualized by autoradiography and quantitated by phosphorimaging of the [^{32}P]phosphate-containing TLC spot reflecting PI 3-P. For calculation of wortmannin-sensitive PI-3K, all values are corrected for PI 3-P radiolabeled in the presence of wortmannin.

Determination of GSK3 activity

GSK3ß activity is determined using immune complex assay with phospho-glycogen synthase peptide 2 (P-GS 2) as a substrate (Eldar-Finkelman et al. 1996; Wang et al. 1994). The GSK3ß immunoprecipitates are washed twice with homogenization buffer and once with assay buffer (20 mM Tris/HCl, pH 7.4, 1 mM DTT) and then suspended in 20 µl of assay buffer containing 0.4 mg/ml BSA, 10 mM $MgCl_2$, 30 µM [γ-^{32}P]ATP (3 000 Ci/mmol, 1 mCi/ml) and 20 µM P-GS 2. After incubation (15 min, 30 °C), the reactions are terminated by addition of 20 µl of 20% trichloroacetic acid and centrifugation (10 000 *g*, 5 min). Then, 15-µl portions of the supernatant are spotted on 2.5 × 3-cm pieces of Whatman P81 phosphocellulose paper. 20 s later, the filters are washed five times with 0.75% phosphoric acid (for at least 5 min each time) and once with acetone. The dried filters are counted for radioactivity in the presence of 5 ml of scintillation fluid (ACS, Amersham Pharmacia Biotech). $^{32}P_i$ incorporation into the negative control peptide (glycogen synthase peptide 2 [Ala21]) is subtracted from values obtained using P-GS 2. No activity was measured with immunoprecipitates using nonimmune IgG. Each activity value is corrected for the amount of immunoprecipitated GSk3ß according to immunoblotting.

Determination of PEPCK activity

PEPCK is measured using the $NaH^{14}CO_3$ fixation assay as described by Noce and Utter (1975); Burcelin et al. (1995) with some modifications. 490 µl of rat liver cytosol is added to 500 µl of reaction buffer containing 150 µmol Tris/acetate (pH 7.2), 5 µmol sodium IDP, 10 µmol $MnCl_2$, 250 µmol KCl, 10 mM DTT, 2 mM GSH, 400/150 µmol $KHCO_3$ and 15 µCi $NaH[^{14}C]O_3^-$ (10 µmol; Amersham-Buchler). The reaction is started by the addition of 10 µl of 0.4 M phosphoenolpyruvate and terminated after 10 min incubation at 25 °C by addition of 1 ml of 6 N HCl and placing the tube on ice. After dilution with 1 ml of deionized water, unreacted CO_2 ($H[^{14}C]O_3^-$) is removed by bubbling with N_2 and CO_2 for 30 min each. The reaction mixture is supplemented with 10 ml of aqueous scintillation cocktail (Beckman ReadySafe) and measured for radioactivity by liquid scintillation counting (Beckman LS6 500). From each value appropriate blanks are subtracted containing the same ingredients but lacking either cytosol or IDP. Under these conditions and up to the maximal amount of cytosol used, the incorporation rates are linear with both cytosol concentration and time during the first 15 min at least.

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K.3.2.12 Insulin receptor substrate

PURPOSE AND RATIONALE

The principal substrate for the insulin and insulin-like growth factor-1 receptors is the cytoplasmatic protein insulin-receptor substrate-1 (IRS-1). IRS-1 undergoes multisite tyrosine phosphorylation and mediates downstream signals by 'docking' various proteins that contain Src homology 2 domains. Insulin derivatives can be characterized by phosphorylation and dephosphorylation kinetics of the insulin receptor, insulin receptor substrate-1 and Shc which is implicated in mitogenic signal transduction. Further members of the insulin receptor substrate family are identified (Fantin et al. 1998).

PROCEDURE

Phosphorylation and dephosphorylation kinetics of insulin receptor and insulin receptor substrates

Rat embryo fibroblasts (Rat-1) stable overexpressing the human receptor isoform A are grown in Dulbecco's modified Eagle's/F12 Mix medium supplemented with 200 nM methotrexate and 10% FCS to confluence in 6 well culture plates and subsequently incubated for 18 h in Dulbecco's modified Eagle's/F12 Mix medium without FCS.

For phosphorylation kinetic studies, cells are incubated with human insulin or the insulin derivatives for various times (0–120 min), subsequently rinsed once with ice-cold buffered saline and solubilized in lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 15 mM $Na_4P_2O_7$, 100 mM NaF, 10% v/v glycerol, 1% v/v Triton X-100, 1 200 trypsin inhibitory units/liter aprotinin, 1 mM PMSF, 1 mM Na_3VO_4, pH 7.5).

For the dephosphorylation kinetics, the cells are incubated with insulin or the test preparations for 3 min and dephosphorylation of the insulin receptor is initiated by dilution of the ligand. The monolayers are then kept for various times at 37 °C. After washing with ice-cold buffered saline, cells are solubilized in lysis buffer. After centrifugation (10 min at 16 000 *g*) the supernatants are diluted in Laemmli buffer (50 mMDTT) and the proteins separated by SDS-polyacrylamide gel electrophoresis.

Tyrosine phosphorylation of IRS-1 in rat diaphragms

After incubation with insulin/compounds, the hemidiaphragms (80–100 mg wet weight) are rapidly liberated from the rib cage, rinsed once with homogenization buffer (25 mM Hepes/KOH, pH 7.4, 140 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM sodium vanadate, 50 mM sodium pyrophosphate, 100 mM NaF, 10 mM glycerol-3-phosphate, 0.2 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 5 µg/ml antipain, 25 µg/ml aprotinin), frozen in liquid N_2 and then homogenized in 2 ml of ice-cold homogenization buffer in a porcelain mortar on ice. After centrifugation (1 500 *g*, 10 min, 4 °C), the fat-free supernatant is supplemented with TX-100 (0.5% final conc.), incubated (30 min, 4 °C) and centrifuged (18 000 *g*, 20 min, 4 °C). The supernatant is precleared by addition of 50 µl of protein A/G-Sepharose (50 mg/ml of the same buffer) and centrifugation (see above). The supernatant is incubated with rabbit anti-rat carboxy-terminal IRS-1 antibodies (Upstate Biotechnology, Lake Placid, USA; 1 : 500) for 2 h at 4 °C and then with protein A/G-Sepharose (5 mg) for 16 h at 4 °C. The precipitates are collected by centrifugation (12 000 *g*, 2 min), washed three times with 1 ml, each, of homogenization buffer containing 1% TX-100, 250 mM NaCl and lacking glycerol and then twice with homogenization buffer lacking TX-100 and all salt ingredients. The immunoprecipitates are suspended in Laemmli sample buffer, heated (95 °C, 5 min) and centrifuged (12 000 *g*, 2 min). The supernatant is subjected to SDS-PAGE (8%) and immunoblotting with either mouse monoclonal anti-phosphotyrosine antibodies (Biomol, Hamburg, Germany; clone 3B12, 1 : 2 000) or anti-IRS-1 antibodies (see above, 1 : 200) using decoration with [^{125}I]protein A (15 µCi/5 ml; Amersham-Buchler, Freiburg, Germany) and autoradiography according to published procedures. Quantitative evaluation is performed by phosphorimaging (Molecular Dynamics, Storm 840).

Tyrosine phosphorylation of the insulin receptor and IRS-1/2/3 in rat adipocytes

Adipocytes (2.8×10^5) incubated with insulin/-compound are separated from the medium by flotation and lysed in 1 ml of chilled buffer containing 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, 50 mM $Na_4P_2O_7$, 10 mM NaF, 10 mM glycerol-3-phosphate, 0.2 mM PMSF, 20 µg/ml leupeptin, 10 µg/ml pepstatin, 25 µg/ml aprotinin by 10 strokes in a loose-fitting Teflon-in-glass homogenizer on ice. The fat-free homogenate, prepared by centrifugation (1500 *g*, 3 min, 15 °C), is supplemented with TX-100 (1% final conc.) and sodium deoxycholate (0.5% final conc.), incubated for 30 min at 4 °C and finally centrifuged (13 000 *g*, 10 min). The soluble fraction is precleared by addition of 100 µl of protein A/G-Sepharose (50 mg/ml of the same buffer) and centrifugation as above. The supernatant is incubated with anti-human insulin receptor β-subunit (from guinea pig; Transduction Laboratories, Lexington, USA, 1 : 200), anti-rat carboxy-terminal IRS-1, anti-rat IRS-2 (from rabbit; Upstate Biotechnology, Lake Placid, USA,

1 : 200) or anti-phosphotyrosine (from rabbit; Biomol, Hamburg, Germany, 1 : 400) or nonimmune rabbit IgG for 4 h at 4 °C and then with protein A/G-Sepharose (5 mg) overnight at 4 °C. The precipitates, collected by centrifugation, are washed three times with 1 ml each of lysis buffer containing 1% TX-100 and lacking glycerol and finally twice with buffer lacking TX-100 and NaCl. The anti-insulin receptor and anti-IgG as well as portions of the anti-IRS-1/2 and anti-phosphotyrosine immunoprecipitates are subjected to SDS-PAGE and immunoblotted using mouse monoclonal anti-phosphotyrosine primary antibodies (1 : 2 000 dilution, Biomol, Hamburg, Germany; clone 3B12) or anti-rat PI 3-kinase antibodies raised against a peptide corresponding to residues 500–519 of p85 protein (from rabbit; 1 : 500, Upstate Biotechnology, Lake Placid, USA) followed by decoration with horseradish peroxidase-coupled secondary antibodies and chemiluminescence detection with the ECL kit (performed according to the instructions from Amersham-Buchler, Braunschweig, Germany), or, alternatively, with [^{125}I]protein A (Amersham-Buchler, Braunschweig, Germany). Quantitative evaluation is performed by phosphorimaging (Molecular Dynamics, Storm 840).

PI 3-kinase activity

Portions of the anti-IRS-1/2 immunoprecipitates (see above) are assayed for PI 3-kinase activity by incubating in 100 µl of 20 mM Tris/HCl (pH 7.0), 50 µM [γ-^{32}P]ATP (5 µCi), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 100 mM NaCl, 2 mM EDTA, 0.5 µM wortmannin (for control incubations, only) containing 10 µg of phosphatidylinositol and 1 µg of phosphatidylserine for 15 min at 22 °C. After addition of 20 µl of 8 M HCl and 160 µl of a 1 : 1 mixture of methanol/chloroform, the extracted phospholipids are resolved by TLC on plates (Silica Gel 60, Merck, Darmstadt) coated with 1% oxalate and developed in chloroform : methanol : water : ammonia (60 : 47 : 11.3 : 3.2, v/v). Radiolabeled PI 3-P (average R_f value of 0.41 under these conditions) is visualized by autoradiography and quantitated by phosphorimage analysis. For calculation of wortmannin-sensitive PI 3-kinase, all values are corrected for PI 3-P radiolabeled in the presence of wortmannin.

Protein kinase B (PKB) activity

Adipocytes (5×10^5) incubated with insulin/compound are washed with KRP-Hepes by flotation and then extracted in 1 ml of 50 mM Hepes/KOH (pH 7.6), 0.2 mM EDTA, 2.2 mM EGTA, 2 mM DTT, 100 mM KCl, 100 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 15% glycerol, 1% Nonidet P-40, 0.5% deoxycholate, 1 µM microcystin, 10 µg/ml pepstatin, 25 µg/ml leupeptin, 1 µg/ml antipain. 900 µl of cell extract is incubated with 10 µl of PKB antiserum (raised in rabbits by immunization with a synthetic peptide corresponding to residues 465–480 of the human PKB sequence) precoupled to 10 mg of protein A-Sepharose in 100 µl of extraction buffer for 2 h at 4 °C. The immunoprecipitates are collected by centrifugation (12 000 *g*, 2 min, 4 °C), washed twice with 1 ml each of PKB assay buffer (20 mM Mops, pH 7.0, 1 mM EDTA, 1 mM EGTA, 10 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, 10 µg/ml aprotinin, 0.1 mM PMSF, 0.01% Brij35, 5% glycerol) containing 1% Nonidet P-40, 0.5 M NaCl and three times with 1 ml each of assay buffer lacking salt and detergent. The immunoprecipitates are finally resuspended in 45 µl of PKB assay buffer containing 10 mM $MgCl_2$, 1 mM DTT, 2.5 µM cAMP-dependent protein kinase inhibitor peptide (IP_{20}) and either the synthetic peptide, Crosstide (100 µM final conc.), which is based on the sequence surrounding the serine phosphorylation site of GSK-3 (Ser21 of GSK-3α and Ser9 of GSK-3β) or alternatively, histone 2B (150 µg/ml). The assay is initiated by the addition of 5 µl of [γ-^{32}P]ATP (50 µM, 4 µCi) and terminated after incubation for 15 min at 30 °C by placing the test tubes on ice. For determination of Crosstide phosphorylation, the 10-µl portions of the samples are adsorbed on p81 phosphocellulose paper and extensively washed as described below for the MBPK assay. The radioactivity associated with the paper is counted by liquid scintillation counting. For determination of histone 2B phosphorylation, the samples are separated on a 12% SDS-PAGE. The autoradiogram is quantitated by densitometry. Exposure times are chosen which guaranteed that the intensity of the bands is linearly related to the quantity of protein contained in the bands.

Protein kinase B (PKB) phosphorylation using immunoblotting

For demonstration of phosphorylation by a size shift in SDS-PAGE analysis, a fat-free homogenate is prepared from adipocytes treated with insulin/compound, supplemented with TX-100 (1% final conc.) and sodium deoxycholate (0.5% final conc.), incubated for 30 min at 4 °C and finally centrifuged (13 000 *g*, 10 min). The supernatant (100 µg protein) is immunoprecipitated with 5 µg of rabbit anti-PKB antibodies coupled to protein G-Sepharose beads. Immune pellets are washed three times with 1 ml each of immunoprecipitation buffer (20 mM Tris/HCl [pH 7.4], 5 mM EDTA, 100 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% TX-100) and three times with 1 ml each of 50 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT. The final immune pellets are suspended in Laemmli sample buffer, heated (95 °C, 2 min) and centrifuged (12 000 *g*, 2 min), and proteins contained in the supernatant separated on SDS-PAGE. PKB is visualized by immunoblotting using a polyclonal anti-rat PKB antibodies from sheep directed

against the N-terminal part of the protein (Santa Cruz Biotechnology, Santa Cruz, USA) and [^{125}I]protein A followed by autoradiography.

Protein kinase B phosphorylation using phosphate incorporation

For demonstration of phosphorylation by $^{32}P_i$ incorporation, isolated rat adipocytes are incubated with [^{32}P]phosphate and then with insulin/compound. A fat-free homogenate is prepared (see above) and centrifuged (150 000 *g*, 60 min, 4 °C). 1-ml portions of the supernatant are immunoprecipitated with 10 µg anti-PKB antibodies coupled to protein G-Sepharose beads. The immunoprecipitates are washed and finally suspended in 50 µl of Laemmli sample buffer (2% SDS), heated (95 °C, 2 min) and centrifuged (12 000 *g*, 2 min). One half of each supernatant is separated by SDS-PAGE and radiolabeled PKB is visualized by phosphorimaging. The other half is diluted with 1 ml of immunoprecipitation buffer containing 10 µg/ml aprotinin, 20 µg/ml pepstatin, 10 µg/ml leupeptin, 1 mM PMSF and centrifuged again (12 000 *g*, 2 min). The supernatant is supplemented with 20 µg of a kit containing four different monoclonal anti-phosphoserine antibodies (Biomol, Hamburg, Germany; clones 1C8, 4A3, 4A9, 4H4) and the monoclonal anti-phosphothreonine antibodies (clone 1E11). After incubation (4 h, 4 °C), the antibodies complexes are precipitates during a 2-h incubation with 50 µl of 50 mg protein A-Sepharose per ml of 50 mM Hepes/KOH (pH 7.4), 0.1% TX-100. The collected immunoprecipitates (12 000 *g*, 2 min) are washed three times with 1 ml each of 50 mM Hepes/KOH (pH 7.4), 150 mM NaCl, 100 mM NaF, 0.2 mM Na_3VO_4, 0.1% SDS, 1% TX-100 and finally twice with buffer lacking TX-100. Phosphoproteins are eluted from the washed precipitates by incubation with 500 µl of 50 mM Hepes/KOH (pH 7.4) containing 50 mM p-nitrophenylphosphate for 30 min at 4 °C. The supernatants obtained by centrifugation are precipitated with TCA. The acetone-washed pellets are suspended in 50 µl of Laemmli sample buffer and analyzed by SDS-PAGE and phosphorimaging of the gel.

PKB serine phosphorylation

One hundred-fifty–200 µg of soluble adipocyte fraction (about 0.5 ml) is supplemented with the same volume of immunoprecipitation buffer (2% NP-40, 25 mM HEPES/KOH pH 7.4, 50 mM NaF, 1 mM Na_3VO_4, 50 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM glycerol-3-phosphate, 1 mM PMSF, 20 µg/ml leupeptin, 10 µg/ml pepstatin, 25 µg/ml aprotinin) and then incubated on ice for 1 h. After addition of 35 µg of anti-PKB antibody and further incubation for 4 h, the mixtures are incubated with 50 µl of protein A-Sepharose (50 mg/ml) overnight with end-over-end rotation at 4 °C. The collected immunoprecipitates (13 000 *g*, 2 min) are washed two times with immunoprecipitation buffer containing 140 mM NaCl, two times with the same buffer lacking NP-40 and finally with immunoprecipitation buffer lacking NP-40. The immunoprecipitated proteins are dissolved in 50 µl of 2-fold Laemmli buffer, separated by SDS-PAGE and immunoblotted with anti-phospho-PKB antibody (1 : 400). Quantitative evaluation is performed by phosphorimaging.

Glycogen synthase-3 (GSK-3) activity

Adipocytes (5×10^5) incubated with insulin/compound are washed with KRP-Hepes by flotation and homogenized in 2 ml of 10 mM Tris/HCl (pH 7.4), 50 mM NaCl, 30 mM $Na_4P_2O_7$, 10 mM glycerol-3-phosphate, 50 mM NaF, 0.1 µM microcystin, 200 µM Na_3VO_4, 0.5 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM PMSF by 10 strokes in a Teflon-in-glass homogenizer. A clear and fat-free cytosol is prepared by three sequential centrifugations at 1 000 *g* for 2 min and twice at 20 000 *g* for 15 min at 4 °C. 100 µl of cytosol is incubated with 5 µl of GSK-3α antiserum (raised in sheep by immunization with a synthetic peptide corresponding to amino acids 471–483 of rat GSK-3α coupled to BSA) for 2 h at 4 °C. The immunocomplexes are precipitated with 2 mg of anti-sheep IgG coupled to agarose in 50 µl of homogenization buffer (under rotation for 2 h at 4 °C) after which the beads are collected by centrifugation (12 000 *g*, 2 min), washed once with 1 ml of 100 mM Tris/HCl (pH 7.4) containing 0.2% Nonidet P-40, 0.5 M NaCl, 0.5 M LiCl, once with 10 mM Tris/HCl and twice with GSK-3 assay buffer (20 mM Hepes/KOH, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 10 mM NaF, 10 mM $Na_4P_2O_7$). The kinase reaction is started by the addition of 50 µl of assay buffer containing 125 µM [γ-^{32}P]ATP (8 µCi), 300 µg/ml P-GS1 peptide and 10 µg/ml heparin to the washed beads. After incubation for 15 min at 30 °C, 10-µl aliquots of the supernatant (obtained by centrifugation as above) are spotted on p81 phosphocellulose papers (1 × 1 cm), which are then washed four times in 75 mM orthophosphoric acid. The radioactivity associated with the papers is measured by liquid scintillation counting. In some experiments recombinant PP2AI (1 µg, Calbiochem, Bad Soden, Germany) is used as substrate instead of P-GS1 or autophosphorylation of GSK-3 is assayed in the absence of exogenous substrate. In these cases the kinase reaction (conditions as above) is terminated by rapid centrifugation (12 000 *g*, 1 min, 4 °C) and separation of the supernatant from the immune pellet. The precipitated (10% TCA for 1 h on ice, 15 000 *g*, 15 min 4 °C) supernatant (for phosphorylated PP2AI) or immune pellet (for phosphorylated GSK-3) is suspended in 50 µl of 2× Laemmli sample buffer, heated (95 °C, 5 min) and

centrifuged (12 000 *g*, 2 min). The supernatants are run on SDS-PAGE and phosphorylated PP2AI and GSK-3 are visualized by autoradiography and quantitated by phosphorimaging.

Glycogen synthase (GS) phosphorylation

Adipocytes are washed twice by flotation in low phosphate medium composed of KRP-HEPES modified to contain 50 µM KH_2PO_4, 2 mM glucose, 2% BSA and then suspended in the same medium (7.5×10^5 cells/ml). 5-ml portions are incubated with [^{32}P]phosphate (0.2 mCi/ml) for 2 h prior to addition of insulin/compound and of 0.5 ml of 50 mM glucose. After incubation for 20 min at 37 °C, the cells are floated by centrifugation (1 000 *g*, 1 min) and the infranatant is aspirated. The cells are suspended in 1.5 ml of cold 50 mM Tris/HCl (pH 7.6), 100 mM KF, 20 mM glycerol-3-phosphate, 10 mM $K_4P_2O_7$, 10 mM EDTA, 1 mM benzamidine, 0.2 mM PMSF and homogenized by 10 strokes using a tight-fitting Teflon homogenizer in the same Eppendorf cup. The homogenate is centrifuged (18 000 *g*, 30 min, 4 °C). 1 ml of the post-mitochondrial supernatant is incubated with 10 µl of GS antiserum raised in guinea pigs by immunization with purified GS from rabbit skeletal muscle [29]. After incubation for 2 h at 4 °C, 50 µl of protein A-Sepharose (10% w/v in TES, see below) is added and the incubation continued overnight. The immunoprecipitates are collected by centrifugation (12 000 *g*, 2 min, 4 °C) and washed twice with 1 ml each of TES (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl) containing 1% TX-100, twice with TES and finally once with 50 mM Tris/HCl (pH 7.4). The beads are suspended in 25 µl of 2xLaemmli sample buffer, heated (95 °C, 5 min) and centrifuged. The supernatant is subjected to SDS-PAGE (8% resolving gel). The amount of [^{32}P] contained in GS is determined by excising the corresponding gel pieces and measuring their radioactivity by liquid scintillation counting.

Glycogen synthase (GS) activity

GS activity is assayed by measuring the incorporation of D-[^{14}C]glucose from UDP-[^{14}C]glucose into glycogen. 10 ml of adipocytes in KRB (about 3×10^5 cells) are incubated with insulin/compound in a shaking water bath under an atmosphere of 5% CO_2 for 30 min at 37 °C. Washed cells are frozen in liquid N_2 and then homogenized in 0.5 ml of 10 mM Tris/HCl (pH 7.5) containing 10 mM EDTA, 150 mM KF, 5 mM DTT by ten strokes in a Teflon-in-glass homogenizer on ice. The homogenate is centrifuged (5 500 *g*, 2 min, 10 °C). The infranatant below the lipid layer is cleared from residual lipids by two additional centrifugations and served as source for GS. The reaction is started by adding 30 µl of the homogenate to 60 µl of a reaction mixture (prewarmed at 30 °C) containing 33 mM Tris/HCl (pH 7.8), 0.2 mM UDP-[U-^{14}C]glucose (4 µCi), 6.7 mg glycogen, 150 mM KF and 0.1 mM/10 mM glucose-6-P. After incubation for 20 min at 30 °C, the reaction is terminated by addition of 2 ml of 66% ethanol, 10 mM LiBr (–20 °C), rapid mixing and filtration over pre-wetted Whatman GF/C glass-fiber discs. The filters are washed 5 times with 5 ml of 66% ethanol each at 25 °C, dried and measured for radioactivity. Blank values determined by adding the homogenate to tubes containing the complete reaction mixture plus ice-cold ethanol are subtracted from the total values each. The fractional velocity is calculated as the ratio of GS activity in the presence of 0.1 mM and 10 mM glucose-6-P. Measurements of GS activity in homogenates from adipocytes which had been incubated in the presence of 0.1 mM glucose instead of 5 mM glucose (the concentration routinely used) did not reveal significant differences with respect to both the activity ratio and the effect of insulin. Presumably, the dilution of the limited amount of glucose-6-P, which accumulates during incubation with 5 mM extracellular glucose, during the subsequent preparation of the homogenate and the GS assay is high enough to prevent allosteric activation of GS. According to our experience incubation of the isolated rat adipocytes in the presence of 5 mM glucose has a positive impact on their viability, in general, and insulin sensitivity (glucose transport, glycogen synthesis), in particular.

Myelin basic protein kinase (MBPK) activity

Adipocytes (2.5×10^5) incubated with insulin/compound are quickly washed with ice-cold KRP-HEPES by flotation and then homogenized in 200 µl of 20 mM HEPES/KOH (pH 7.2), 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 40 mM p-nitrophenylphosphat, 50 mM glycerol-3-phosphate, 5 mM $Na_4P_2O_7$, 20 mM NaF, 0.2 mM PMSF, 25 µg/ml leupeptin, 25 µg/ml aprotinin by 10 strokes of a rotating tight-fitting Teflon pestle in the same Eppendorf cup at 4 °C. The homogenate is centrifuged (2 000 *g*, 5 min, 4 °C). The infranatant below the fat cake is removed and centrifuged (30 000 *g*, 5 min, 4 °C). After careful removal of the residual fat layer, the supernatant is used for measurement of MBPK by combining 5-µl portions (about 10 µg protein) with 45 µl of kinase buffer (50 mM glycerol-3-phosphate, pH 7.3, 5 mM $Na_4P_2O_7$, 10 mM NaF, 0.5 mM EDTA, 15 mM $MgCl_2$, 2 mM DTT, 4.4 mM protein kinase inhibitor peptide) containing 0.5 mg/ml myelin basic protein (MBP). The kinase reaction is initiated by the addition of 5 µl of 1 mM [γ-^{32}P]ATP (700 mCi/mmol). Following a 10-min incubation at 30 °C, reactions are terminated by spotting 10 µl of the reaction mixture onto p81 phosphocellulose papers (Whatman), which are immediately immersed in

0.85% orthophosphoric acid under stirring and washed once in 95% ethanol for 5 min. Papers are dried and ^{32}P is quantitated by liquid scintillation counting.

Mitogen-activated protein kinase (MAPK) activity

Adipocytes (5×10^5) incubated with insulin/compound are washed with KRP-HEPES by flotation and then lysed in 1 ml of 50 mM HEPES/KOH (pH 7.2), 100 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5 mM Na_3VO_4, 40 mM p-nitrophenylphosphate, 10 mM glycerol-3-phosphate, 10 mM NaF, 0.2 mM PMSF, 25 µg/ml leupeptin, 25 µg/ml aprotinin by incubation for 30 min on ice and vigorous vortexing three times for 10 s at 10-min intervals. Lysates are cleared by centrifugation (12 000 *g*, 10 min, 4 °C). The infranatant below the fat cake is removed using a syringe, recentrifuged and used for measurement of MAPK by addition of 250-µl portions to 250 µl of protein A Sepharose (50 mg/ml of 50 mM Hepes/KOH, pH 7.2, 100 mM NaCl), which had been precoupled with 10 µl of rabbit anti-p42MAPK antiserum (raised against rat p42MAPK) and 5 µl of rabbit anti-p44MAPK antiserum (raised against the C-terminal 14 amino acids of rat p44MAPK), and incubation for 2 h at 4 °C. The beads are collected by centrifugation (12 000 *g*, 2 min, 4 °C), washed three times with 1 ml each of lysis buffer and three times with 1 ml each of 10 mM HEPES/KOH (pH 7.4), 10 mM $MgCl_2$ and then resuspended in 20 µl of the same buffer containing 2 mg MBP per ml. The kinase assay is initiated by the addition of 20 µl of 40 mM HEPES/KOH (pH 7.4), 200 µM [γ-^{32}P]ATP (25 mCi/mmol), 40 mM $MgCl_2$, 20 mM NaF, 0.2 mM Na_3VO_4. After incubation for 30 min at 30 °C, the reaction is terminated by the addition of 20 µl of 4× Laemmli sample buffer. Samples are subjected to SDS-PAGE using a 15% resolving gel. The gel is dried and the radiolabeled MBP is quantified by phosphorimaging.

Preparation of detergent-insoluble complexes

Many components of the insulin signaling cascade (e.g. insulin receptor) are localized in special areas of the plasma membrane of adipocytes and myocytes, the so-called caveolae (Rothberg et al. 1992; Anderson 1993a and b, Kurzchalia et al. 1994; Parton 1996; Anderson 1998). Caveolae serve as special signaling compartment keeping the corresponding components in a basal inactive state but competent for future activation (Lisanti et al. 1994; Couet et al. 1997; Schlegel et al. 1998; Okamoto et al. 1998). Thus, the analysis of insulin signaling processes may be facilitated by using caveolae instead of total cell extracts. Caveolae can be isolated simply as detergent-insoluble complexes since they resist solubilization by non-ionic detergents in the cold due to their high content of glyco(sphingo)lipids and cholesterol (Brown and Rose 1992; Brown and London 1997). Furthermore, certain sulfonylureas seem to accumulate at caveolar plasma membrane regions of adipose cells due to spontaneous intercalation between glycosyl-phosphatidylinositol (GPI) lipids. Thereby a non-receptor tyrosine kinase attached to the cytoplasmic face of caveolae and the GPI-specific phospholipase is activated which then leads to phosphorylation of IRS-1/2 initiating metabolic insulin signaling (see above; Müller and Geisen 1996; Müller and Frick 1999; Müller 2000).

For preparation of detergent-insoluble complexes, 9×10^7 adipocytes are lysed on ice in 2 ml of isotonic lysis solution (25 mM Tris/HCl pH 7.4; 140 mM NaCl, 1% NP-40, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2 µM leupeptin, 5 µM pepstatin, 2 mM EDTA, 5 mM iodoacetamide by 10 strokes of a Dounce homogenizer and five 10-s bursts of a bath sonicator. After removal of nuclei by centrifugation (3 000 *g*, 3 min), the supernatant is mixed on ice 1 : 1 with 80% sucrose in lysis solution. The sample (1 ml of detergent cell lysate in 40% sucrose) is overlaid with 5 ml of 30% sucrose in lysis solution followed by 5 ml of 5% sucrose in lysis solution and finally by 1 ml of lysis solution lacking sucrose. After centrifugation (20 h, 40 000 rpm, 4 °C, Beckman SW41 rotor), the gradient is fractionated into 12 fractions of identical volume. A light-scattering band is confined to fraction 2 and 3 (from the top of the gradient) corresponding to the 5–30% sucrose interface.

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K.3.2.13 Effect of insulin on glycosyl-phosphatidylinositol-specific phospholipase C

PURPOSE AND RATIONALE

A low molecular weight substance was identified that was released from hepatic plasma membranes in response to insulin which may be a second messenger to insulin (Saltiel and Cuatrecasas 1986). The generation of this substance by insulin can be reproduced by addition of a phosphatidylinositol-specific phospholipase C. This enzyme is known to selectively hydrolyze phosphatidylinositol and release from membranes several proteins that are covalently linked to a glycosyl-phosphatidylinositol (GPI) anchor (Low and Saltiel 1988; Low 1989; Ferguson and Williams 1988; Cross 1990). In addition to the released diacylglycerol, phosphoinositolglycans (PIG) are therefore considered as precursor for an insulin specific second messenger (Larner 1988; Romero et al. 1988; Saltiel 1990).

During the past two decades a large body of evidence has accumulated that PIG molecules can exert some insulin-mimetic activity on glucose and lipid metabolism on insulin target cells *in vitro*. They have been demonstrated to stimulate glucose transport, glycolysis, glycogen synthesis, lipid synthesis and to inhibit glucagon-induced gluconeogenesis and isoproterenol-induced lipolysis in isolated or cultured muscle, fat and liver cells to a partial degree (Saltiel 1990; Romero and Larner 1993; Varela-Nieto et al. 1996; Jones and Varela-Nieto 1998). The molecular basis of these PIG actions seems to rely on the appropriate modulation of the activity of the corresponding key metabolic and

regulatory enzymes/proteins, like pyruvate dehydrogenase, glycogen synthase, glycerol-3-phosphate acyltransferase, glycogen phosphorylase, protein kinase A, cGMP-inhibitable cAMP-specific phosphodiesterase, protein phosphatase 1 and 2C (Varela-Nieto et al. 1996; Jones and Varela-Nieto 1998). This partial insulin-mimetic metabolic activity together with the mechanistic findings that (I) some metabolic insulin actions can be inhibited by anti-PIG antibodies in intact BC_3H-1 myocytes (Romero et al. 1990), (II) insulin stimulation of glycogen synthesis is blocked in mutant K562 erythroleukemia cells which are totally deficient in GPI synthesis (Lazer et al. 1994), (III) isolated PIG can exert acute hypoglycemic activity in normal and streptozotocin diabetic rats (Fonteles et al. Asplin et al. 1993), (IV) PIG can be isolated from normal subjects and in reduced amounts from NIDDM patients (Shashkin et al. 1997), suggests but does not prove the involvement of GPI structures, GPI cleavage or PIG molecules in metabolic insulin signalling. The partial insulin-mimetic activity of PIG structures in cellular and cell-free assay systems (Farese 1990; Gaulton and Pratt 1994) in combination with the possibility of the generation of PIG molecules from GPI structures by regulated lipolytic or combined lipolytic/proteolytic cleavages (Müller et al. 1993 and 1994; Romero et al. 1988; Movahedi and Hooper 1997) prompted P. Cuatrecasas, J. Larner, M. G. Low, J. Mato, and A. Saltiel in the late 80s to assign PIG molecules a function as soluble mediators of metabolic insulin action (Low and Saltiel 1988; Romero et al. 1988; Larner 1987; Saltiel et al. 1988; Mato 1989). According to this hypothesis, PIG molecules are generated in response to insulin through lipolytic cleavage of free GPI lipids and/or GPI proteins by a phospholipase/protease at the extracellular face of the plasma membrane of insulin-sensitive cells and are then transported into the cytosol, where they directly affect key metabolic enzymes and/or their regulatory proteins in an allosteric fashion.

In fact, it has been demonstrated that insulin stimulates a GPI-specific phospholipase C in cultured and isolated adipocytes as well as muscle cells in a concentration-dependent and rapid fashion. The GPI-PLC cleaves glycosyl-phosphatidylinositol (GPI) lipids, which exist either in free form or as membrane anchors covalently bound to GPI-anchored proteins (GPI proteins), cell surface ectoproteins of eucaryotic cells, embedded in the outer leaflet of the plasma membrane by the covalently linked GPI moiety (Nosjean et al. 1997), such as the cAMP-binding ectoprotein, Gce1, of rat and mouse adipocytes (Müller et al. 1994). Many cell types including adipose and muscle cells harbor an endogenous GPI-specific PLC (GPI-PLC) which upon activation causes the release of the protein moiety of GPI proteins from the cell surface into the cellular environment due to lipolytic cleavage of its GPI anchor (Saltiel et al. 1986; Romero et al. 1988). In isolated and cultured adipocytes certain GPI proteins, such as lipoprotein lipase, are released from the cell surface into the incubation medium by the action of an endogenous GPI-PLC which is activated by both insulin and Amaryl (Romero et al. 1988; Müller et al. 1993 and 1994; Movahedi 1997).

PROCEDURE

Glycosyl-phosphatidylinositol-specific phospholipase C is assayed as the conversion of glycosyl-phosphatidylinositol-anchored plasma membrane protein lipoprotein lipase (LPL) from its amphiphilic version (containing the intact glycosyl-phosphatidylinositol anchor) into its hydrophilic version (containing the lipolytically cleaved glycosyl-phosphatidylinositol anchor).

Metabolic labeling of rat adipocytes

Adipocytes are cultured, as described by Marshall et al. (1984). After collagenase digestion, the cells (3×10^7 cells/ml) are washed three times with 4 ml/ml cell suspension of Dulbecco's modified Eagle's medium (DMEM) containing 1% BSA, 5% fetal bovine serum, 100 units of penicillin/ml, 100 µg of streptomycin/ml and 1 mg of Fungizone/ml and finally suspended in the same buffer supplemented with 5 mM glucose at $3–5 \times 10^5$ cells/ml. The radiolabelling is performed by the addition of 250 µCi of myo-[^{14}C]inositol or 400 µCi of [^{14}C]stearic acid to 20 ml of cell suspension and incubation for 14 h or 1 h, respectively. Subsequently, the cells were concentrated by flotation (1 000 *g*, 2 min, 25 °C), washed three times with 20 ml of DMEM, 0.5% BSA, 1 mM myo-inositol or 10 mM stearic acid per ml of packed cells and finally suspended in buffer for preparation of plasma membranes. Labeling with [^{35}S]methionine is performed according to Lawrence et al. (1986) with following modifications: Adipocytes are washed and suspended in methionine-free DMEM containing the same ingredients as indicated above. After addition of [^{35}S]methionine (1 mCi/10 ml of cell suspension, approximately 1 000 Ci/mmol), the cells are incubated for 1 h at 37 °C, then floated by centrifugation (1 000 *g*, 2 min) and washed twice in DMEM containing 10 mM unlabeled methionine.

The cells are incubated with standard insulin or test compounds at various concentrations for 30 min at 37 °C. Subsequently, the cells are concentrated by flotation (1 000 *g*, 2 min, 25 °C), washed three times with DMEM, 0.5% BSA, 1 mM *myo*-inositol per ml of packed cells and finally suspended in buffer for preparation of plasma membranes. Plasma membranes are isolated and subjected to partitioning between a TX-114 phase and aqueous phase (Bordier 1981; Pryde and

Phillips 1986). Metabolically labeled lipoprotein lipase (LPL) is analyzed in the aqueous phase by immunoprecipitation with anti-LPL antibodies and fluorography (Müller et al. 1993).

Assay for insulin-specific phospholipase

(Müller and Bandlow 1991; Müller et al. 1993)

Adipocytes are photoaffinity-labeled with 8-N_3-[^{32}P]cAMP to specifically label the regulatory subunit of a cAMP-dependent ectoprotein kinase of the plasma membrane.

The labeling is performed in 24-well culture plates. Cells are washed twice with 2 ml of DMEM containing 1% BSA, 1 mM EDTA, 100 mM NaCl, without glucose, serum and antibiotics. To each monolayer, 10 µCi 8-N_3-[^{32}P]cAMP (0.5 nM) or 2 µCi 8-N_3-[^{3}H]cAMP in 0.5 ml of phosphate-buffered saline (250 mM sucrose, 1 mM EDTA, 50 mM KCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM IBMX, 1 mM dithiothreitol, 1 mM AMP, 0.1 mM PMSF) is added. The cells are incubated for 30 min at 4 °C and then irradiated with UV light at 254 nm (8 000 µW/cm^2) at a distance of 2 cm for 2 min. Subsequently, the cells are washed twice with 2 ml of the same buffer containing 1 mM unlabeled cAMP instead of 8-N_3-[^{32}P]cAMP and then for incubation with the test compound supplemented with 1 ml of high glucose DMEM, 10% FCS, 0.5% BSA 50 units/ml penicillin, 50 µg/ml streptomycin sulfate.

The cells are separated from the medium by centrifugation on silicon oil. The cells as well as the medium are subjected to TX-114 partitioning. The detergent phase is separated from the aqueous phase by centrifugation. The aqueous phases are removed, dried and subjected to SDS gel electrophoresis and autoradiography. The labeled bands are excised and counted for radiography.

The assay measures the insulin-stimulated release of a glycosyl-phosphatidylinositol anchored plasma membrane protein into the medium. It has been proposed that phosphoinositolglycan structures derived from glycolipids or glycosyl-phosphatidylinositol modified plasma membrane proteins (e.g. alkaline phosphatase) by insulin-dependent lipolytic cleavage (phospholipase C) act as second messengers for a variety of insulin-dependent metabolic reactions. Therefore, the insulin-stimulated phospholipase determined by this assay may reflect an early step in the signal transduction cascade.

EVALUATION

Concentration-response curves are established using various concentrations of standard (human insulin) and the test compounds, e.g. sulfonylureas, allowing calculation of ED_{50} values and potency ratios.

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K.3.2.14
Preparation of caveolae and detergent-insoluble glycolipid-enriched raft domains (DIGs)

PURPOSE AND RATIONALE

Caveolae are flask- or bulb-shaped invaginations (50 to 100 nm diameter) of the plasma membrane abundantly expressed in terminally differentiated cells, such as endothelial, epithelial, muscle and adipose cells which play important roles in intracellular transport and signaling processes (Anderson 1998; Engelman et al. 1998; Okamoto et al. 1998; Parton 1996). They are characterized by a unique lipid composition consisting of cholesterol, glycosphingolipids and glycosyl-phosphatidylinositol (GPI). These detergent-resistant (in the presence of 1% Triton X-100 at 4 °C for 1 h) liquid-ordered domains purified from mammalian cells and tissues (by means of sucrose gradient centrifugation due to low buoyant density) are referred to as detergent-insoluble glycolipid-enriched rafts (DIGs) (Brown and London 1997 and 1998) and have been demonstrated by several independent methods to exist in living cells (Harder et al. 1998; Rietveld and Simons 1998). Caveolins, 21- to 25-kDa integral membrane proteins with three isoforms (Glenney 1992; Rothberg 1992; Parton 1996; Scherer et al. 1997), are the structural and marker proteins of caveolae and are dramatically enriched in DIGs. DIGs lacking caveolin may represent the biogenetic precursors for caveolae (precaveolae) and DIGs containing caveolin can be considered as the biochemical correlate of caveolae (Okamoto et al. 1998; Schlegel et al. 1999).

Caveolae and DIGs harbor a number of components of various intracellular signal transduction pathways including G protein-coupled receptors, heterotrimeric G proteins, receptor tyrosine kinases (Src family tyrosine kinases), components of the Ras-mitogen-activated protein kinase (MAPK) pathway, protein kinase C isoforms, and endothelial nitric oxide synthase (eNOS) (Schlegel et al. 1999; Shaul and Anderson 1998; Smart et al. 1999). As a consequence, they may function as locations for the direct physical interaction of signaling elements where the cross-talk between the corresponding signaling pathways takes place (Okamoto et al. 1998). Lisanti and coworkers demonstrated direct binding of a common domain within caveolin, the caveolin

scaffolding domain (CSD) to the corresponding caveolin binding domain (CBD) of a variety of signaling proteins which are thereby kept in a basal inactive state, however competent for future activation (Couet et al. 1997a; Ju et al. 1997; Mineo et al. 1998; Moffett et al. 2000). The inhibitory interaction between caveolin and signaling molecules should be accessible for modulation in response to extracellular/intracellular signals. Activation of signaling pathways engaging CBD-harboring signaling molecules requires their relief from binding to/inhibition by caveolin both in short and long term. The molecular mechanism for the long term response may be based on the regulation of caveolin gene expression. Consistent with this hypothesis, caveolin-1 mRNA and protein expression as well as the number of caveolae are dramatically diminished upon cell transformation by activated oncogenes, such as H-Ras (Koleske et al. 1995). Some putative physiological mechanisms for short term regulation of the caveolin-signaling component interaction have been described (Feron et al. 1996; Garcia-Cardena et al. 1997), among them the coordination of the interaction of receptors and a variety of downstream signal transducing molecules that localize to the plasma membrane following cell stimulation (Müller and Frick 1999; Okamoto et al. 1998; Smart et al. 1999). Agonist stimulation has been demonstrated to result in redistribution of receptors for contractile agonists (e.g. bradykinin, de Weerd et al. 1997; acetylcholine, Feron et al. 1997), hormones (e.g. insulin, Gustavsson et al. 1999; angiotensin II, Ishizaka et al. 1998) and growth factors (e.g. EGF, Couet et al. 1997) as well as of downstream elements (e.g. PKCα, Mineo et al. 1998; and rhoA, Taggert et al. 2000) to caveolin-containing subcellular fractions. A pathway for insulin activation of glucose transport has been identified in insulin-sensitive cells which involves DIGs/caveolae and the in- and out- movement of signaling proteins (Baumann et al. 2000; Ribon et al. 1998). Thus, DIGs/caveolae seem to harbor signaling components for insulin-stimulated glucose transport and regulate their activity by interaction with caveolin and additional caveolar structural proteins that may serve as scaffolding proteins for concentrating and activating/inhibiting specific insulin signaling components.

However, small molecules, which manage to modulate the interaction of caveolin/caveolae with CBD-containing signaling proteins and, in consequence, the activation state of the corresponding downstream signaling cascades have not been described so far with one important exception (Scherer and Scherer 1997). A study by Müller et al. (2001) demonstrated that in isolated rat adipocytes, the non-receptor tyrosine kinases, $pp59^{Lyn}$ and $pp125^{Fak}$, are down-regulated by localization in caveolae/binding to caveolin and upregulated by release from caveolae/dissociation from caveolin in response to phosphoinositolglycans (PIG), degradation products of GPI membrane protein anchors (Jones and Varela-Nieto 1999; Müller and Frick 1999), and the sulfonylu-rea drug, glimepiride (Langtry and Balfour 1998). The short term redistribution of these kinases from caveolae/DIGs induces insulin-independent activation of the me-tabolic insulin signaling cascade. These findings suggest that the concentration of signaling proteins at cave-olae/caveolin may be regulated by diverse physiological and pharmacological stimuli thereby integrating cross-talk to various signal transduction pathways.

PROCEDURE

For studying signal transduction processes which involve caveolae, these structures are isolated as DIGs by biochemical methods (carbonate or detergent extraction) from primary or cultured differentiated cells, such as muscle and fat cells, after incubation with external stimuli. DIGs can be prepared either directly from total cells, or total cell lysates or (preferably) isolated (purified) plasma membranes. The use of DIGs from total cells or lysates may obscure subtle changes in signaling since the (considerable) fraction of DIGs present in membranes of the Golgi apparatus (where their biogenesis takes place) may respond differently (or not at all) toward external stimuli compared to the plasma membrane DIGs. Subsequent analysis for the presence in/cofractionation with caveolae, direct interaction with caveolin and changes in activity of signaling proteins in response to the external stimuli requires the methods of enrichment/purification of DIGs, photoaffinity labeling (of GPI proteins), coimmunoprecipitation with caveolin (of signaling proteins), immunoblotting (of signaling proteins) and immune complex kinase assays (in case of kinase activity of signaling proteins).

Preparation of DIGs from total cells

Carbonate method

Washed adipocytes (0.5×10^7 cells) are suspended in 2 ml of sodium carbonate buffer (0.5 M Na_2CO_3, pH 11) containing protease inhibitors and homogenized sequentially using a loosely fitting Dounce homogenizer (10 strokes) and a sonicator (3×20 s bursts). The homogenate (2 ml) is then adjusted to 45% sucrose by addition of 2 ml of 90% sucrose, 50 mM MES/KOH (pH 6.5), 150 mM NaCl (final pH of the mixture 10.2). A discontinuous sucrose gradient is formed by overlaying this solution with 4 ml of 35% sucrose and 4 ml of 5% sucrose, both in the same buffer containing 0.25 M Na_2CO_3. After centrifugation (see above), 0.85-ml gradient fractions are collected to yield a total of 14 fractions. The individual gradient fractions are pooled into

DIGs (Fr. 4–7) and non-DIG areas (Fr. 10–14). The membranes from each pooled gradient fractions obtained by either method are diluted 2- to 3-fold with 25 mM MES (pH 6.5), 150 mM NaCl, 1% TX-100, collected by centrifugation (50 000 *g*, 30 min, 4 °C) and resuspended in non-dissociating buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 0.5 mM EGTA, 1 mM sodium orthovanadate, 50 mM NaF and protease inhibitors) or dissociating buffer (= non-dissociating buffer supplemented with 60 mM β-octylthioglucoside, 0.3% deoxycholate) as indicated, incubated (1 h, on ice) and used for (co)immunoprecipitation, immunoblotting or photoaffinity labeling.

Detergent method

Washed adipocytes (3.5×10^6) are suspended in 1.5 ml of lysis buffer (25 mM MES, pH 6.0, 150 mM NaCl, 5 mM EDTA, 1% TX-100, 0.2 mM sodium orthovanadate, and protease inhibitors) and incubated for 20 min on ice. The cells are lysed with 10 strokes in a manual Teflon-in-glass homogenizer over the course of 1 h at 4 °C. The lysate is centrifuged (1 300 *g*, 5 min) to pellet unbroken cells, cellular debris, nuclei and large unsoluble material. One ml of the postnuclear supernatant is subjected to sucrose gradient centrifugation by mixing with an equal volume of 85% sucrose, 25 mM MES (pH 6.0), 150 mM NaCl, 5 mM EDTA at the bottom of a 12-ml centrifuge tube which is overlayed with 5.5 ml of 35% sucrose, and then 3.5 ml of 5% sucrose in the same medium. After centrifugation (230 000 *g*, Beckman SW41 rotor, 18 h, 4 °C), 0.9-ml fractions are collected from top to bottom, and termed fraction Fr. 1, 2, 3, etc. The bottom fraction is Fr. 12. Fr. 5 appears as a white, light-scattering band under illumination located at 5–7% sucrose at the 35-% sucrose interface. The DIGs contained in Fr. 5 are pelleted by dilution of the sucrose with 5 volumes of 50 mM HEPES/KOH con-taining protease inhibitors and centrifugation (200 000 *g*, 2 h).

Preparation of plasma membranes appropriate for subsequent generation of DIGs

Basal or stimulated rat adipocytes (5×10^7 cells) are washed once with PBS containing 2 mM sodium pyruvate by flotation (200 *g*, 2 min) and aspiration of the infranatant and immediately homogenized in 10 ml of lysis buffer (25 mM Tris/HCl, pH 7.4, 0.5 mM EDTA, 0.25 mM EGTA, 0.25 M sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 25 mM glycerol-3-phosphate and 1 mM sodium orthovanadate, supplemented with protease inhibitors [10 µg/ml leupeptin, 2 µM pepstatin, 10 µg/ml aprotinin, 5 µM antipain, 5 mM iodo-acetate, 100 µM phenylmethylsulfonyl fluoride, 4 mM benzamidine]) using a motor-driven Teflon-in-glass homogenizer (10 strokes with a loosely fitting pestle) at 22 °C. The following procedures are performed at 4 °C (Müller and Wied 1993). After centrifugation (1 500 *g*, 5 min), the postnuclear infranatant is separated from the fat cake, and the pellet fraction (containing adipocyte ghosts and cell debris) by suction with a needle. For preparation of plasma membranes, the postnuclear infranatant is centrifuged (12 000 *g*, 15 min). The pellet is suspended in 10 ml of homogenization buffer and re-centrifuged (1 000 *g*, 10 min). The supernatant is centrifuged (12 000 *g*, 20 min). The washed pellet is suspended in 1 ml of homogenization buffer, layered onto a 5-ml cushion of 38% (w/v) sucrose, 25 mM Tris/HCl (pH 7.4), 1 mM EDTA, and centrifuged (110 000 *g*, 1 h). The membranes at the interface between the two layers (0.5 ml) are removed by suction, diluted with four volumes of homogenization buffer, and layered on top of an 8-ml cushion of 28% Percoll, 0.25 M sucrose, 1 mM EDTA, 25 mM Tris/HCl (pH 7.0). After centrifugation (45 000 *g*, 30 min), the plasma membranes are withdrawn from the lower third of the gradient (0.5 ml) with a Pasteur pipette, diluted with 10 volumes of homogenization buffer and centrifuged (200 000 *g*, 90 min). The washed pellet is suspended in the same buffer at 0.5 mg protein/ml.

Preparation and purification of DIGs from plasma membranes

Plasma membranes (200 µg) are pelleted (200 000 *g*, 90 min) and suspended in 1.5 ml of 0.5 M Na_2CO_3 (pH 11) containing protease inhibitors and sonicated (3×30-s bursts with 1-min intervals on ice, Branson B-12, power stage 4). The suspension is then adjusted to 45% sucrose in 15 mM Mes/KOH (pH 6.5), 75 mM NaCl, 0.25 M Na_2CO_3, overlayed with 2 ml each of 35, 25, 15, and 5% sucrose in the same medium, and centrifuged (230 000 *g*, Beckman SW41 rotor, 18 h). The light-scattering band of flocculent material just below the 15–25% sucrose interface is collected as DIGs using a 19-gauge needle and a syringe (about 1.5 ml).

Coimmunoprecipitation

Total cell lysates (25–50 µg protein) or DIGs in non-dissociating buffer (10–15 µg protein) are precleared (1 h, 4 °C) with protein G/A-Sepharose (50 mg/ml) in a total volume of 100 µl and then supplemented with appropriate antibodies ($pp125^{Fak}$: 2 µg/sample; IRS-1: 1 : 50 dilution; IRS-2: 10 µg/sample; $pp59^{Lyn}$: 5 µg/sample; caveolin: 0.7 µg/sample; IRβ: 1 : 175 dilution) preadsorbed on protein G-Sepharose (monoclonal antibodies) or protein A-Sepharose (rabbit antibodies) in a total volume of 100 µl. After incubation (4 h, 4 °C, end-over-end rotation) and centrifugation (3 000 *g*, 2 min,

4 °C), the collected immune complexes are washed twice with 1 ml each of immunoprecipitation buffer (50 mM HEPES/KOH, pH 7.4, 500 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate) containing 0.2% Nonidet P-40 and 0.3% deoxycholate, then twice with 1 ml each of immunoprecipitation buffer containing 150 mM NaCl and 0.2% Nonidet P-40 and once with 1 ml of immunoprecipitation buffer lacking salt and detergent and finally suspended in 50 µl of Laemmli buffer (4% SDS, 115 mM Tris/HCl, pH 6.8, 1 mM EDTA, 10% glycerol, 4 mg/ml bromophenol blue) supplemented with 1.2% β-mercaptoethanol (except for anti-caveolin immunoprecipitates), heated (95 °C, 2 min) and centrifuged. The supernatant samples are analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE; 4–12% Bis-Tris precast gel, pH 6.4, morpholinoethanesulfonic acid/SDS running buffer under reducing conditions. The centrifugation conditions for collection of the protein A/G Sepharose-bound immune complexes, do not lead to sedimentation of (non-dissociated DIGs) to any significant degree according to immunoblotting with anti-caveolin antibody. The coimmunoprecipitation of proteins with caveolin from non-dissociated DIGs is specific for DIG-associated components, such as the GPI protein, Gce1, and the dual-acylated non-receptor tyrosine kinase, $pp59^{Lyn}$, since the use of non-immune IgG instead of anti-caveolin antibody or of dissociated DIGs and anti-caveolin antibody do not result in immunoprecipation of Gce1 and $pp59^{Lyn}$.

Immunoblotting

Immunoblotting is performed as described above with minor modifications. After SDS-PAGE and transfer of the proteins to polyvinylidene difluoride membranes (2 h, 400 mA in 20% methanol, 192 mM glycine, 25 mM Tris, 0.005% SDS), the blocked membrane (1 h in 20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20, 0.1% Brij, 0.01% NP-40 and with 1% ovalbumin and 1% BSA (anti-phosphotyrosine, anti-$pp59^{Lyn}$, anti-IRβ) or with 5% non-fat dried milk (anti-caveolin, anti-IRS-1/2) is incubated (2 h, 25 °C) with antibodies against IRS-1 (1 : 500), IRS-2 (1 : 750), caveolin (1 : 2 000), $pp59^{Lyn}$ (1 µg/ml) or IPβ (2 µg/ml) diluted in the same medium, and then washed five times with the same medium. After incubation of the membranes (1 h, 25 °C) with horseradish peroxidase-coupled goat anti-mouse IgG antibody or goat anti-rabbit IgG antibody diluted in the appropriate blocking buffer (1 : 5 000 or 1 : 2 500) and subsequent washing with detergent-containing (two times) and detergent-free (two times) buffer (20 mM Tris/HCl, pH 7.6, 150 mM NaCl), the labeled proteins are visualized by the enhanced chemiluminescence method.

Immune complex kinase assays

Immune complex kinase assays are performed as described above with minor modifications. $pp59^{Lyn}$ immune complexes are suspended 30 µl of kinase buffer (50 mM HEPES/KOH, pH 7.4, 100 mM NaCl, 1.25 mM $MnCl_2$, 12.5 mM $MgCl_2$, 1.25 mM EGTA, 0.5 mM DTT, 1 mM Na_3VO_4) containing [γ-^{32}P]ATP (final conc. 40 µM, 0.2 mCi/ml) or 1 mM ATP and incubated ($pp59^{Lyn}$: 15 min, 22 °C) in the presence of 1 µg heat-denatured enolase. Phosphorylation is terminated by addition of 10 µl of four-fold concentrated Laemmli buffer and boiling. The phosphoproteins are separated on SDS-PAGE (10% Bis-Tris resolving gel, morpholinopropanesulfonic acid/SDS running buffer) and analyzed for phosphotyrosine by phosphorimaging ([γ-^{32}P]ATP) or immunoblotting (ATP). Under these conditions the kinase reactions are linear with time for the assay period. IRS-1 immune complexes were assayed for phosphatidylinositol-3 kinase (PI-3'K) by incubating (10 min, 22 °C) in 50 µl of 20 mM Tris/HCl (pH 7.0), 50 µM [γ-^{33}P]ATP (5 µCi), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 100 mM NaCl, 2 mM EDTA, 0.5 µM wortmannin (for control incubations, only) containing 10 µg of phosphatidylinositol (PI) and 1 µg of phosphatidylserine. After addition of 10 µl of 8 M HCl and 160 µl of a 1 : 1 mixture of methanol/chloroform, the extracted phospholipids are resolved by TLC on plates coated with 1% oxalate and developed in chloroform: methanol:water:ammonia (60 : 47 : 11.3 : 3.2, v/v). Radiolabeled PI 3-P is quantitated by phosphorimage analysis. For calculation of wortmannin-sensitive PI-3'K, all values are corrected for PI 3-P radiolabeled in the presence of wortmannin.

Photoaffinity labeling of plasma membranes and DIGs for detection of the GPI protein, Gce1

Solubilized plasma membranes and DIGs (5–10 µg protein) are incubated (30 min, 4 °C) with 50 µCi 8-N_3-[^{32}P]cAMP (0.5 nmol) in 50 µl of 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM isobutylmethylxanthine, 1 mM DTT, 1 mM AMP, and protease inhibitors in the wells of microtiter plates (96-formate) and then irradiated with UV light (254 nm, 8 000 µW/cm^2) at a distance of 0.5 cm for 1 min (Müller et al. 1993, 1994a and b). Subsequently, the photoaffinity labeling reaction is quenched by addition of 100 µl of the same buffer containing 10 mM cAMP. Gce1 is precipitated (5% trichloroacetic acid, 1 h on ice, 10 000 *g* for 15 min) and solubilized in sample buffer for SDS-PAGE. Protein concentration is determined using the BCA protein determination kit from Pierce (Rockford, IL). Autoradiographs and direct photoimages are processed and quantified by computer-assisted video densitometry using the Storm 860 Phosphor-Imager system

(Molecular Dynamics, Gelsenkirchen, Germany). Figures of autoradiographs and photoimages are constructed using the Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA). The recovery in the amounts of immunoprecipitated protein has to be corrected (data on fold- or % stimulation) for the amount of protein actually applied onto the gel as revealed by homologous immunoblotting. Each experiment/incubation should be performed with different batches of adipocytes with two to four independent immunoprecipitation/kinase assay/immunoblotting analyses.

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K.3.3 Isolated diaphragm of rats or mice[3]

GENERAL CONSIDERATIONS

The determination of insulin based on the stimulation of glucose uptake by the isolated diaphragm from mice and rats has been used by many investigators. Formerly, the method has been used for determination of serum insulin (Groen et al. 1952; Randle 1954; Vallance-Owen 1954, 1960; Wright 1957; Willebrands et al. 1958; Antoniades 1961; Moody and Felber 1964; Stock 1973) but the epididymal fat pad assay (see above) turned out to be more sensitive and the immunological methods based on the findings of Yalow and Berson (1960) are more specific. Nevertheless, the isolated diaphragm of rats or mice is still useful to study the effects of insulin and insulin-mimetic substances on muscle tissue.

Instead of the isolated rat diaphragm, Adolfsson et al. (1967) recommended the use of the intact levator ani muscle of rats to study the incorporation of labelled proline into protein.

K.3.3.1 Glycogen synthesis

PROCEDURE

Male Sprague Dawley rats weighing 70–100 g are used. The animals are sacrificed during anesthesia and the diaphragms are carefully removed, spread out and divided into two equal pieces. The hemidiaphragms are incubated in Krebs-Henseleit buffer gassed with carbogen (95% O_2/5% CO_2) with 5 µM [U-^{14}C] glucose (0.5 µCi/ml), insulin or the compound to be tested. After 30 min, the hemidiaphragms are blotted on tissue, frozen in liquid nitrogen, and ground in a porcelain mortar and pestle chilled with liquid nitrogen. Samples of the powdered tissue are weighed and dissolved by heating for 45 min at 100°C in 30% KOH (1 ml/100 mg tissue) before ethanol is added to a concentration of 70%. After 4 h at –20°C, the samples are centrifuged at 2 000*g* for 10 min. The glycogen pellets are washed 3 times with 70% ethanol before the amount of ^{14}C labelled glycogen is determined by liquid scintillation counting. Total glycogen is determined according to Lowry and Passo-neau (1972) after hydrolysis to glucose (1 N HCl at 100°C for 3 h). The incorporation of [^{14}C]glucose into radiolabeled glycogen is normalized for the total amount of glycogen.

EVALUATION

The concentration dependence of [U-^{14}C] glucose uptake and conversion into glycogen by insulin or insulinomimetic compounds is determined.

The isolated diaphragm of rats or mice is the preferable organ to study the effect of insulin and substances with insulin-mimetic effects, such as sulfonylureas, on muscle tissue or the influence of denervation (Standing and Foy 1970; Smith and Lawrence 1984; Ishizuka et al. 1990; Hothersall et al. 1990).

K.3.3.2 Glucose transport

Intact washed rat diaphragms are incubated (30 min, 37 °C) in HEPES-buffered saline (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 0.5 mM sodium pyruvate, 1.5 mM KH_2PO_4, pH 7.4) under constant bubbling with 95% O_2/5% CO_2. The diaphragms are then washed two times with the same buffer lacking glucose and further incubated (30 min) in 5 ml of glucose-free buffer in the presence of test compounds or insulin. Glucose transport is initiated by addition of 50 µl of 10 mM 2-[1-^{3}H]deoxyglucose (10 µCi/ml) in the absence or presence of 25 µM cytochalasin B (control). After 15 min, the diaphragms are rinsed four times with ice-cold buffer containing 10 mM glucose, 25 µM cytochalasin B, blotted with filter paper and homogenized. Portions of the homogenate are used for protein determination. One-ml portions of the supernatant of a centrifugation at 10 000 *g* for 15 min are mixed with 10 ml scintillation cocktail and counted for radioactivity.

Specific glucose transport (dpm/mg of protein) is calculated as the difference between diaphragm-associated radioactivity measured in the absence (total

[3] Contributions by G. Müller.

uptake) and presence of cytochalasin B (non-specific uptake). Under these experimental conditions, transport is linear for 30 min.

K.3.3.3 Glycogen synthase

Glycogen synthase is assayed according to Oron and Larner (1979), Guinovart et al. (1979), Altan et al. (1985) with the following modifications: Intact hemidiaphragms are dissected from male Wistar rats and incubated in DMEM (10 ml/hemidiaphragm) with constant bubbling of $O_2 : CO_2$ (95 : 5). For treatment with test compounds or insulin, the hemidiaphragms are incubated (37 °C) in the same medium plus 5 mM glucose. For preparation of a homogenate, the diaphragms are blotted and frozen in liquid nitrogen. The frozen diaphragms (pool of four) are manually ground in a porcelain mortar and then homogenized at 0 °C in 10 vol of 25 mM Tris/HCl (pH 7.4), 100 mM NaF, 5 mM EDTA, 0.1 mM PMSF. The homogenate is centrifuged (10 000 *g*, 20 min). The supernatant is used for the glycogen synthase assay.

After addition of 10 µl of diaphragm homogenate to 200 µl of 25 mM Tris/HCl (pH 7.4), 50 mM NaF, 10 mM EDTA (pre-incubated at 30 °C) containing either 0.1 or 10 mM glucose-6-phosphate, the reaction is initiated by supplementing 0.2 mM [U-^{14}C]UDP-glucose (10 µCi) and terminated after 15 min by the addition of 2.5 ml of ice-cold 66% ethanol and filtration over pre-wetted Whatman GF/C glass fiber disks. The filters are washed, dried and counted for radioactivity. Blanks are assayed by adding the homogenate to tubes containing the complete reaction mixture plus ice-cold ethanol. The fractional velocity as parameter for the portion of glycogen synthase active *in vivo* (l-form) toward the total enzyme contents (l- + d-forms) at the time point of homogenization is calculated as ratio between the activities measured at 0.1 (l-form) and 10 mM glucose-6-phosphate (l- + d-forms).

The concentration-dependence of sulfonylurea stimulated glycogen synthase and the potentiation of insulin were studied by Müller et al. (1994).

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K.3.4 Binding assays

K.3.4.1 Immunoassay

PURPOSE AND RATIONALE

The first description of an immunoassay of endogenous plasma insulin in man has been given by Yalow and Berson (1959, 1960), Yalow et al. (1960) providing evidence that the bioassays hitherto being used (isolated rat diaphragm, epididymal fat pad tissue) measure insulin-like activity but not true insulin levels in blood. Since that time, the method has been used and modified by many investigators, e.g., Grodsky and Forsham (1960), Morgan and Lazarow (1963), Melani et al. (1965, 1967), Wright et al. 1968). Survey on the radioimmunoassay of insulin have been given by Ditschuneit and Faulhaber (1975), Freedlender et al. (1984).

PROCEDURE

Immunization

Semisynthetic or biosynthetic human insulin is used as immunogen and as standard. Formerly, porcine insulin has been used since Yalow and Berson (1960) and subsequently many other authors have shown that antisera raised against porcine insulin react identically with human and porcine insulin. Guinea pigs weighing 350–450 g are injected subcutaneously with 0.4 ml of an emulsion of 5 mg human insulin dissolved in 1.0 ml 0.01 N HCl and 3.0 ml complete Freund's adjuvant (Difco Laboratories). For boostering, 0.2 ml of an identically prepared emulsion is injected in monthly intervals. Fourteen days after the third booster injection, the animals are slightly anesthetized and 8–10 ml blood are withdrawn by cardiac puncture. Boosting is continued at monthly intervals and the animals are bled 2 weeks following each booster injection.

Antiserum

The optimal antiserum titer for use in the radioimmunoassay is determined using conditions identical to those employed in routine immunoassays. The percentage binding of 1 μU ^{125}I insulin is determined for dilutions of antisera ranging from 10^3 to 10^6 fold. The steepness of the antisera dilution curve is a measure of the affinity of the antiserum and therefore the potential sensitivity of the radioimmunoassay. Antisera with the steepest slopes, but not necessarily the highest titer, are selected for further study. The selected antisera dilutions are then run in an immunoassay using a full range of standards. A reduction in the percent ^{125}I-insulin bound to antibody from 50% (in the absence of unlabeled insulin) to 45% (in the presence of unlabeled insulin) (B/Bo = 0.9) is a reasonable measure of assay sensitivity.

Preparation of ^{125}I-insulin

Most investigators use the "chloramine-T procedure" to iodinate insulin.

The reaction is carried out in a 20 ml glass vial in an ice-bath with continuous magnetic stirring. To 2.5 ml 0.05 M phosphate buffer, pH 7.5, 2.0 mCi Na^{125}I, and 15 μl of a 1 mg/ml insulin solution are added. Then, 0.5 ml of a chloramine T (50 mg/ml) solution is added dropwise over the course of 1 min. After 10 min, 0.7 ml of a freshly prepared sodium metabisulfite solution (50 mg/ml in 0.05 M phosphate buffer, pH 7.5) is added. One ml of this reaction mixture is transferred to 10 ml 2% bovine serum albumin for determination of specific activity. In order to absorb unreacted ^{125}I and damaged products 2.0 g 20–50 mesh AG 1X-8 resin (BioRad Laboratories) are added (equilibrated in 1 ml 0.05 M phosphate buffer, pH 7.5, containing 0.1 mg/ml thiomerosal and 20 mg/ml crystalline bovine serum albumin). The reaction mixture is stirred for 10 min, decanted from the resin and diluted to a concentration of less than 25 μC/ml in a solution of 0.8 M glycine, 0.2 M NaCl, 0.05 M phosphate (pH 7.5), and 2.5 mg/ml crystalline BSA. The final solution is stored in multiple aliquots at –70 °C.

Assay procedure

The antibody-bound ^{125}I-insulin can be separated from free ^{125}I-insulin in various ways, such as by paper electrophoresis, as originally described by Yalow and Berson (1960), or by a two-antibody system, as described by Morgan and Lazarow (1963), Starr et al. (1979). In this method, the soluble insulin-anti-insulin complex is precipitated by an anti-guinea pig serum antibody.

The following procedure is recommended (Freedlender et al. 1984):

- A buffer is prepared from a solution of 8.25 g boric acid and 2.70 g NaOH dissolved in 1 l water. After dissolving 5.0 g of purified bovine serum albumin, pH is adjusted with concentrated HCl to 8.0.
- In disposable plastic tubes, 10 × 75 mm, the following volumes are added:
  100 μl serum or standard
  900 μl buffer
  100 μl 1 mU ^{125}I-insulin in assay buffer
  100 μl guinea pig anti-insulin antiserum diluted in assay buffer (at a concentration to bind 50% of the ^{125}I-insulin in the absence of unlabeled hormone)
- The mixture is incubated at 4 °C for 72 h. Then, the following solutions are added:

100 µl normal guinea pig serum diluted 1 : 400 in the assay buffer
100 µl rabbit anti-guinea pig globulin serum diluted in assay buffer

- The mixture is again incubated at 4 °C for 72 h and then centrifuged at 4 °C and 2 000 *g* for 20 min. The supernatant is decanted and radioactivity counted in the precipitate for 5 min.

Calculation

Counts in the nonspecific binding tubes are subtracted from counts in all other tubes. Data are linearized using an unweighted logit-log transformation (Rodbard and Frazier 1975). Micro-units insulin in a logarithmic scale are plotted against the ratio B/Bo ^{125}I-insulin on a logit scale. The range of B/Bo between 0.4 and 0.9 is the most suitable for determination of insulin concentration in plasma.

CRITICAL ASSESSMENT OF THE METHOD

The immunoassay of insulin as described by Yalow and Berson (1960) has been a break-through for many immunological assays of peptide hormones and other drugs. At present, not only guinea pig anti-insulin antisera, but also complete RIA kits are available from a number of commercial firms.

MODIFICATIONS OF THE METHOD

Cam and McNeill (1996) published a sensitive radioimmunoassay optimized for reproducible measurement of rat plasma insulin. Relatively small volumes (25 µl) of plasma from control, diabetic, and fasted rats can be assayed reproducibly with charcoal in the final separation step.

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K.3.4.2 Insulin receptor binding

PURPOSE AND RATIONALE

Insulin receptor binding studies have been performed with various animal tissues and isolated cells as well as with cells of human origin. Human adipocytes can be used to study simultaneously insulin receptor binding and metabolic effects of insulin (Hjøllund 1991). The binding tests are of value to characterize newly synthesized insulin derivatives (Schwartz et al. 1987; Ribel et al. 1990; Vølund et al. 1991; Robertson et al. 1992).

PROCEDURE

Subcutaneous adipose tissue (about 4–5 g) is obtained from the abdomen of patients undergoing gastroenterological surgery. Patients suffering from any endocrine or metabolic disorder or taking drugs known to affect metabolism have to be excluded. Other exclusion criteria are impaired glucose tolerance measured by determination of fasting blood glucose and the 2 h value after a 75 g oral glucose load. The adipose tissue is finely chopped and incubated for 90 min at 37 °C in a HEPES buffer (pH 7.4), containing human serum albumin (25 g/l) and collagenase (0.5 g/l). The isolated adipocytes are subsequently washed five times in a HEPES buffer containing 50 g/l human albumin. The diameters of adipocytes are measured at 200-fold magnification using an eyepiece micrometer. Surface and volume are calculated for every cell diameter.

Insulin receptor binding studies with isolated human adipocytes are performed in a 300 µl cell suspension containing about 1×10^5 cells/ml in a HEPES buffer (10 mmol/l HEPES, 50 g/l human serum albumin, (pH 7.4) at 37 °C. The iodine labelled ligand ([^{125}I]Tyr^{A14}-monoiodinated insulin, specific activity about 350 mCi/mg) in a final concentration of 20 pmol/l

is incubated with increasing amounts of unlabeled human insulin and the insulin derivative to be tested. The reaction is stopped by adding 10 ml of chilled 0.154 mol/l NaCl and subsequent centrifugation with silicone oil (Pederson et al. 1981; Zeuzem et al. 1984). Non-specific binding is measured by incubating tracer in the presence of a large excess of unlabelled insulin.

For association studies the ^{125}I-labelled ligand is incubated for various times (1 to 240 min) and the reaction is terminated as described above. At each time point, the non-specific binding is measured and subsequently subtracted from the corresponding data for total binding.

Dissociation rates are determined by first incubating isolated human adipocytes at 37 °C with either [^{125}I]Tyr^{A14}-insulin or the test compound labelled in the same position for 90 min to achieve steady-state binding conditions. Each incubation mixture is then centrifuged for 60 s The adipocytes are rapidly washed twice by diluting with buffer to the original volume at 4 °C and the centrifugations and aspirations are repeated. After the third aspiration, the cells are diluted to the original volume with buffer alone or native insulin or the insulin derivative to be tested at a final concentration of 0.2 µmol/l at 22 °C. At this hormone concentration a maximal effect of ^{125}I-insulin dissociation is reported (Podlecki et al. 1984; DeMeyts et al. 1976). The reaction is stopped at various times between 10 and 180 min and cell-associated radioactivity is determined.

EVALUATION

Results are expressed as percentage specific binding per 10 cm^2 plasma membrane surface area (Olefsky 1976; Pedersen et al. 1982).

MODIFICATIONS OF THE METHOD

Koch and Weber (1981) described the preparation of insulin receptors from rat liver membranes. The membranes are prepared from normal male or female Sprague-Dawley rats weighing about 130 g and starved 12–15 h before decapitation. The livers are cut into small pieces on ice. The whole procedure is performed at 4 °C. Five g of liver in 1 000 ml 0.01 M phosphate buffer (pH 7.4) containing 0.25 M sucrose are homogenized first with an Ultra-Turrax® and then in a glass-Teflon homogenizer with 10 strokes up and down at 100 rotations per min. The homogenate is centrifuged for 10 min at 600 *g*. The pellets are discarded and the supernatant centrifuged at 12 000 *g* (30 min) and 40 000 *g* (80 min). The pellet is suspended in phosphate buffer and again centrifuged at 40 000 *g* for 60 min. This washing step is repeated twice. The pellets are resuspended in 30 ml 0.01 M phosphate buffer. At least 8 concentrations of test substance and standard are used for the binding test.

Heterogeneity of insulin receptors in different tissues has been emphasized by Gammeltoft (1988), Breiner et al. (1993).

Simonescu et al(1985) described a radioreceptor assay of insulin using **human erythrocytes**.

Kergoat et al. (1988) studied the properties of the liver insulin receptor and the activity of the insulin receptor tyrosine kinase in rats with non-insulin-dependent diabetes induced by neonatal streptozotocin administration.

Hurrell et al. (1989) prepared solubilized purified insulin receptors from livers of Zucker fatty rats and Sprague-Dawley rats with dietary obesity.

Olichon-Berthe et al. (1994) studied insulin receptor dephosphorylation by phosphotyrosine phosphatases obtained from liver and muscle of goldthioglucose-treated insulin-resistant obese mice.

Standaert et al. (1984) studied the development of high affinity insulin receptors and insulin-stimulated responses in the differentiating **nonfusing muscle cell line BC3H-1**.

Bornfeldt et al. (1991) studied receptor binding and growth promoting effects of insulin, insulin analogues and insulin-like growth factors in **cultured rat aortic smooth muscle cells**.

DeMeyts (1976) described insulin and growth hormone receptors in human cultured lymphocytes and **peripheral blood monocytes**.

Wyse and Chang (1981) characterized insulin receptors in the established **Chinese hamster kidney epithelial cell line CHK-AC$_{E\text{-}100}$**.

Klein et al. (1986) studied insulin activation of insulin receptor tyrosine kinase in intact rat adipocytes.

Weiland et al. (1990) described antagonistic effects of a covalently dimerized insulin derivative on insulin receptors in **3T3-L1 adipocytes**.

Levy and Belsky (1990) studied insulin receptors in cultured **hepatoma HepG2 cells**.

Drejer et al. (1991) studied receptor binding and tyrosine kinase activation by insulin analogues in human hepatoma HepG2 cells. The kinetic studies showed that differences in affinities between analogues were due to differences in both dissociation and association constants.

Markussen et al. (1991) purified the **soluble ectodomain of the human insulin receptor**, produced in transfected baby hamster kidney cells, by affinity chromatography on immobilized insulin.

Kurose et al. (1994) studied the binding of an insulin derivative to **human insulin receptor overexpressed on a transfected Chinese hamster ovary cell line**.

Schäffer et al. (1993) produced chimeric insulin/type I insulin-like growth factor receptors to study the interaction of a hybrid insulin/insulin-like growth factor-I analog.

Schumacher et al. (1993) **generated chimeric receptors** in which the structurally defined subdomains of the insulin receptor and insulin growth factor-I receptor a-subunits were exchanged for their respective receptor backbone structures.

Burke et al. (1980) showed a divergence of the *in vitro* biological activity and receptor binding affinity (liver plasma membranes and fat cells) of a synthetic insulin analogue, [21-asparaginamide-A]insulin.

Müller et al. (1991) showed that hyperglycemia induces an acute inhibition of the insulin receptor tyrosine kinase in rat-1 fibroblasts overexpressing the human insulin receptor. This effect is paralleled by translocation of several protein kinase C (PKC) isoforms, and it can be prevented by PKC inhibitors. The glucose-induced inhibition of the insulin receptor kinase could also be prevented by incubation with troglitazone (Kellerer et al. 1994).

Shah et al. (1995) described a method for electronic microscopic visualization of insulin binding and insulin translocation into the cytoplasm and nuclei of intact H35 hepatoma cells using covalently linked nanogold-insulin.

Whitcomb et al. (1985a,b) described an *in vivo* **radioreceptor assay** for identification of tissue insulin receptors.

Awasthi et al. (1994) used ^{99m}Tc-insulin for distribution studies in animals.

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K.4
Assays of other glucose regulating peptide hormones

K.4.0.1
Bioassay of glucagon

PURPOSE AND RATIONALE

Glucagon is a 29-amino-acid, single-chain polypeptide which is secreted by the α cells in the islets of Langerhans. It is synthesized from preproglucagon, a 180-amino-acid precursor (Bell et al. 1983). Proglucagon is processed in islet and intestinal cell lines (Tucker et al. 1996). Glucagon interacts with a 60-kDa glycoprotein receptor on the plasma membrane of target cells (Sheetz and Tager 1988).

A biological assay of glucagon is described in the British Pharmacopoeia 1988. The potency of glucagon is estimated by comparing its hyperglycemic activity with that of the standard preparation of glucagon using the rabbit blood sugar assay as performed for insulin determinations.

PROCEDURE

Rabbits of either sex, weighing 1.8 to 2.8 kg are maintained under uniform conditions and an adequate uniform diet for at least one week. Forty-eight hours before the beginning of the test, each rabbit is injected with 1 ml of cortisone acetate injection. The animals are deprived of food, but not water, from 16 h before each test day until the withdrawal of the last blood sample on that day. The rabbits are randomly distributed into 4 groups of at least 6 animals.

The standard preparation to be used is the 1st International Standard for Glucagon, porcine, established in 1973, consisting of freeze-dried porcine glucagon with lactose and sodium chloride (supplied in ampoules containing 1.49 Units), or another suitable preparation the potency of which has been determined in relation to the International Standard.

The entire contents of one ampoule of the standard preparation are reconstituted with 2 ml of saline solution, acidified to pH 3.0 with hydrochloric acid and diluted with the same solvent to a convenient concentration, for example 100 milliUnits per ml. Two dilu-

tions are made containing 24 and 6 milliUnits per ml, respectively, and at the same time two dilutions are made of the preparation being examined. The rabbits are injected subcutaneously with doses of 1 ml of each of the four solutions, giving the doses in random order following a twin cross-over design on two consecutive days at the same time each day. At 20 and 60 min after injection, a blood sample is taken from a marginal ear vein of each rabbit.

Blood glucose concentrations are determined using a suitable method, such as the glucose-oxidase procedure.

EVALUATION

The result of the assay is calculated by standard statistical methods using the means of the two blood glucose levels found for each rabbit.

MODIFICATIONS OF THE METHOD

Glucagon can be determined by radioimmunoassay (Unger 1959; Harris et al. 1978; von Schenk 1984). Commercial kits are available.

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K.4.0.2 Receptor binding and *in vitro* activity of glucagon

PURPOSE AND RATIONALE

The binding of glucagon to its receptor is assayed with rat liver plasma membranes (Neville 1968; Pohl et al. 1971; Goldstein and Blecher 1976). Displacement of ^{125}I-labeled glucagon is measured for synthetic glucagon analogues in comparison to natural glucagon. Cyclic AMP formation as the first step in glucagon action on liver is measured as a bioassay in liver plasma membranes.

PROCEDURE

Preparation of membranes

Male Sprague-Dawley rats weighing 160–200 g are decapitated and the livers rapidly removed and trimmed of fat and connective tissue. The pooled livers are placed on a pre-chilled glass plate and chopped finely with a stainless steel blade. Ten-gram portions of the mincate are suspended in 35 ml of ice-cold 1 mM $NaHCO_3$ and homogenized in a loose fitting Dounce homogenizer. Two homogenates are combined, brought to 500 ml with ice-cold medium, stirred magnetically for 5 min, and finally filtered once through two layers of cheesecloth. The filtrate is centrifuged at 1 500 *g* for 10 min. Supernatant fluid is aspirated to waste using a serum pipette attached to a water pump. Homogenizations and centrifugations are continued until all of the tissue has been processed. The pooled pellets are again homogenized. The suspension is adjusted to 44 ±0.1% (wt/wt) sucrose solution by the addition of 70% sucrose solution. Sucrose gradients are prepared in 1 × 3.5-in. tubes by pipetting 26 ml of the tissue suspension followed by an overlay of 13 ml of 42.3 ±0.1% sucrose. Centrifugation is carried out at 95 100 *g* for 2 h. The float containing the plasma membranes can be removed by pinching the tube slightly above the float, then lifting off the float with a spoon-shaped Teflon-coated spatula. These floats are transferred to a preweighed plastic centrifuge tube (50 ml), and the well-mixed suspension centrifuged at 40 000 *g* for 30 min. Following aspiration of the supernatant to waste, the tubes are reweighed in order to estimate the yield of plasma membranes. After addition of an equal volume of medium, the pellet is aspirated repeatedly through a 20-gauge needle fitted to a syringe. The plasma membrane suspension is distributed in 0.2 or 1.0 ml aliquots to screwcap plastic vessels for storage in liquid nitrogen.

Radioiodination of glucagon

Three nmol glucagon are allowed to react with a 1.0 nmol sample of carrier-free $Na^{125}I$ (2.0 mC) in the presence of 1.5 nmol chloramine-T, added at a regular interval of 30 s (0.5 nmol each time). Reaction is terminated by addition of 0.5% sodium metabisulfite solution (Hagopian and Tager 1983). By chromatographic purification (Jørgensen and Larsen 1972), fractions of 2 ml are collected, and the monoiodinated glucagon as determined by reverse phase HPLC is stored at –20 °C for receptor binding assays.

Receptor binding

Membrane suspensions adjusted to 50 µg protein in 400 µl of Tris-HCl buffer (25 mM, pH 7.5, with 1% BSA) are incubated for 10 min with 50 µl peptide solution (Tris-HCl buffer) and 50 µl of [^{125}I]glucagon (1 000 000 cpm, 25 fmol). The samples are then filtered through Oxoid filters and washed three times with 1 ml of Tris-HCl buffer. The radioactivity retained in the filters is counted by a γ-counter.

Adenylate cyclase assay

The assay is carried out with a membrane suspension containing 25–30 µg protein in a volume of 0.1 ml of Tris-HCl buffer 25 mM, pH 7.5 containing BSA 1%, ATP 1 mM, $MgCl_2$ 5 mM, cAMP 1 nM, (containing 10 000 cpm of [^{3}H]cAMP), GTP 10 mM, phosphocreatine 20 mM, with 4×10^6 cpm [α^{32}P]ATP and 0.72 mg/ml creatinine phosphokinase (100 U/ml). Assays are run in triplicate.

EVALUATION

Results are expressed as the percentage inhibition of [^{125}I]glucagon specific binding for receptor binding assays. For adenylate cyclase assays the results are expressed as percent potency relative to the maximal stimulation by glucagon which is defined as 100%.

MODIFICATIONS OF THE METHOD

Azizeh et al. (1995, 1997) synthesized and tested a glucagon antagonist and multiple replacement analogues of glucagon by adenylate cyclase assay according to Lin et al. (1975) and receptor binding assay according to Wright and Rodbell (1979).

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K.4.0.3 Glucagon-like peptide I

PURPOSE AND RATIONALE

Several intestinal peptides have been described to have insulinotropic or incretin activity, e.g. GIP (gastric inhibitory peptide, glucose-dependent insulin releasing peptide) (Creutzfeldt 1985; Baer and Dupré 1989; Volz et al. 1995). More recently, glucagon-like peptide-1 (7-37) or the (7-36)amide is described as a new incretin (Kreymann et al. 1987; Fehmann et al. 1989, 1990, 1991a, 1992, 1995; Holz et al. 1993). The insulinotropic activity has been confirmed in diabetic and non diabetic subjects (Gutniak 1992; Nathan et al. 1992; Nauck et al. 1993; Ørskov 1993). The insulin stimulatory effect of glucagon-like peptide-1(7-36)amide is glucose-dependent (Göke et al. 1993a). The peptide does not only stimulate insulin release but also inhibits glucagon secretion (Komatsu et al. (1989). Cloning and functional expression of the rat (Thorens 1992) and human (Dillon et al. 1993) glucagon-like peptide1 (GLP-1) re-

ceptor has been achieved. The sequence of glucagon-like peptide-1 (7-36)amide is completely conserved in all mammalian species studied, implying that it plays a critical physiological role. Intracerebroventricular administration of GLP-1 powerfully inhibits feeding in fasted rats, which is blocked by the GLP-1-receptor antagonist exendin(9-39) (Turton et al. 1996).

Peptides isolated from reptile venoms, like exendin-4, were found to have similar activity as glucagon-like peptide-1(7-36)amide (Göke et al. 1993b; Adelhorst et al. 1994; Schepp et al. 1994). Analogues and antagonists of glucagon-like peptide-1(7-36)-amide have been synthesized and evaluated in pharmacological experiments (Watanabe et al. 1994; Hjorth and Schwartz 1996; Montrose-Rafizadeh et al. 1997). Besides insulin release from perfused pancreas (see K.6.1.1), the receptor binding according to Göke and Conlon (1988) on rat insulinoma-derived cells (RINm5F cells), the cAMP formation and the insulin release were studied (Göke et al. 1989a,b).

PROCEDURE

Binding studies with RINm5F cells

The RINm5F cell line is derived from a radiation-induced insulin-producing rat tumor (Gazdar et al. 1980). The RINm5F cells are grown in plastic culture bottles (Praz et al. 1983). They are detached from the surface of the bottles before the experiment using phosphate-buffered saline (136 mmol NaCl/l, 2.7 mmol KCl/l, 8.1 mmol Na_2HPO_4/l, 1.5 mmol KH_2PO_4/l, pH 7.3) containing 0.7 mmol EDTA/l, and centrifuged at 100 *g* for 5 min. The pelleted cells are resuspended in an incubation buffer (2.5 mmol Tris-HCl/l, 120 mmol NaCl/l, 1.2 mmol $MgSO_4$/l, 1.5 mmol KCl/l, and 15 mmol CH_3COONa/l, pH 7.4) containing 1% human serum albumin, 0.1% bacitracin and 1 mmol EDTA/l. Approximately 3×10^6 cells/tube are incubated for 5 min at 37 °C, followed by the addition of unlabelled peptide (final concentration range from 10 pmol to 1 mmol) and radiolabelled tracer (approximately 40 000 c.p.m.). Iodination of the glucagon-like peptide-1(7-36)amide is carried out using the lactoperoxidase method. The total volume of incubation is 0.3 ml. After incubation for 60 min, aliquots (200 µl) of the cell suspensions are centrifuged (11 500 *g* for 2 min) through an oil layer (dibutylphthalate:dinonylphthalate; 10 : 4, v/v). Cell-surface associated radioactivity in the pellet is counted using a γ-counter (e.g. Gamma 5 500, Beckman).

Determination of cAMP production from RINm5F cells

Approximately 1×10^6 cells in 0.45 ml buffer (113 mmol NaCl/l, 4.7 mmol KCl/l, 1.2 mmol KH_2PO_4/l, 10 mmol HEPES/l, 2.5 mmol $CaCl_2$/l and 1.2 mmol $MgSO_4$/l; pH 7.4) containing 1% human serum albumin are pre-incubated for 10 min at 37 °C, and then incubated for 10 min after the addition of 2 µl 3-isobutyl-1-methyl-xanthine (50 mmol/l) in order to prevent the breakdown of cAMP. The reaction is then started by the addition of 50 µl of a peptide solution dissolved in the above buffer (final concentration range from 10 pmol/l to 1 µmol/l). After incubation for 10 min at 37 °C, the reaction is stopped by the addition of 200 µl 12% trichloroacetic acid. The reaction mixture is sonicated for 30 s at 25 W (e.g., Heat system, Ultrasonics) and centrifuged (11 500 *g* for 2 min). HCl (25 µl; 1 mol/l) is added to 0.5 ml supernatant. Trichloroacetic acid dissolved in the supernatant is removed by diethyl ether (3×1 ml) and the resulting supernatant is stored at –40 °C until cAMP assays being performed by use of a RIA kit.

Determination of insulin release from RINm5F cells

One day prior to the experiment, approximately 1×10^6 cells are seeded into 24-well test plates. At the time of the experiment, the culture medium is aspirated, and the cells are washed with 1 ml of a modified Krebs-Ringer bicarbonate buffer containing 10 mmol HEPES/l, 5 mmol $NaHCO_3$/l, 0.5% BSA, and 8.3 mmol glucose/l (pH 7.4). After pre-incubation for 30 min at 37 °C with the above buffer, glucagon-like peptide-1(7-36)amide and its analogues dissolved in the above buffer, are added (final concentration range from 10 pmol/l to 1 µmol/l) and incubated for 30 min at 37 °C. The incubation is stopped by aspiration of the buffer which is stored at –40 °C until measurement of insulin by radioimmunoassay. After the aspiration, the cells are washed with the phosphate-buffered saline used in the binding study, and dissolved in 0.5 ml NaOH (0.1 mol/l). After overnight incubation, the resulting solution is collected for assay of cellular protein content.

EVALUATION

Displacement curves of ^{125}I-labelled glucagon-like peptide-1(7-36)amide are established for various concentrations of glucagon-like peptide-1(7-36)amide and its analogues as well as dose-response curves for cAMP production and insulin release. The data are analyzed by analysis of variance followed by group comparisons (Duncan's multiple comparisons).

MODIFICATIONS OF THE METHOD

Similar experiments were performed with other insulinoma cell lines such as HIT-T15 cells and βTC-1 cells (Fehmann and Habener 1991a,b, 1992). Active glucagon-like peptide-1(7-36)amide receptors have been solubilized from RINm5F plasma membranes (Göke et al. 1992).

Valverde et al. (1993) reported the presence and characterization of glucagon-like peptide-1(7-36) amide receptors in solubilized membranes of rat adipose tissue.

Jehle et al. (1995) found that in RINm5F insulinoma cells GLP-1 potently stimulates insulin secretion and insulin content and improves insulin receptor binding.

Fehmann et al. (1991b) evaluated the effects of helodermin, a peptide isolated from the lizard Gila monster, in isolated rat pancreas.

Schepp et al. (1996) studied binding of oxyntomodulin, an intestinal peptide hormone derived from proglucagon, to the receptor for glucagon-like peptide-1 (7-36)NH_2.

Van Delft et al. (1997) developed a sensitive radioimmunoassay for C-terminally amidated forms of glucagon-like peptide-1.

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K.4.0.4 Insulin-like growth factors

PURPOSE AND RATIONALE

Three different lines of research have led to the discovery of insulin-like growth factors (Froesch et al. 1985): Growth hormone does not stimulate growth processes by itself but induces factors, named sulfation factors or **somatomedins**, that mediate the message of growth hormone (Salmon and Daughaday 1957; Nevo 1982; Laron 1999). Pierson and Temin (1972) extracted factors from serum with multiplication-stimulating activity (MSA) for chicken fibroblasts in the cell culture and with non-suppressible insulin-like activity. Serum exerts insulin-like effects on insulin target tissues such as muscle and adipose tissues to a much greater extent than it can be expected on the basis of the insulin content in serum. The activity of factors other than insulin in the epididymal fat pad assay was proven by the persistence of serum insulin-like activity after pancreatectomy (Steinke et al. 1962). Since this activity could not be suppressed by insulin antibodies, the factors were called **'non-suppressible insulin-like activity'** or **NSILA**.

They were finally identified as **insulin-like growth factors I and II** (Rinderknecht and Humbel 1978a,b) structurally related to insulin. These polypeptide hormones are present in serum in high concentrations but are bound to specific carrier proteins (Zapf et al. 1975). Most of IGF and also the IGF-binding protein are synthesized and secreted, but apparently not stored by the liver (Schwander et al. 1983).

Insulin-like growth factor 1 (IGF-1) is a polypeptide of 70 amino acids, whose function of is more or less clarified (Cascieri et al. 1988; Moxley et al. 1990; Salamon et al. 1991; Schmitz et al. 1991; Vikman et al. 1991; Laron 1999). The metabolic activity of IGF-1 is regulated by at least 6 IGF-binding proteins, the most important being IGFBP-3. The effects of growth hormone on protein metabolism are mediated by IGF-1, whereas these 2 hormones are antagonistic in their effects on insulin and some aspects of lipid metabolism. IGF-1 has been shown to improve glycemic control and to reduce insulin requirements in both IDDM and NIDDM (Simpson et al. 1998). One important clinical use is replacement therapy in primary IGF-1 deficiency, such as Laron syndrome, which is characterized by dwarfism due to primary GH resistance or insensitivity (Laron 1999). IGF-1 improves bone healing *in vivo* (Kobayashi et al. 1996). A truncated variant of human IGF-1 with the tripeptide Gly-Pro-Glu absent from the N-terminus, has been isolated from bovine colostrum, human brain and porcine uterus which is about 10-fold more potent than IGF-1 at stim-ulating hypertrophy and proliferation of cultured cells (Ballard et al. 1999).

The function of insulin-like growth factor II is less defined (Roth 1988). IGF-II has an insulin-like effect *in vitro* and *in vivo* (Shizume et al. 1996; Burvin et al. 1998). IGF-II, administered acutely, affects glucose homeostasis in a manner very similar to insulin, probably via the insulin receptors, although with significantly lower potency.

Two subtypes of IGF receptors exist which are different from the insulin receptor and different in their affinity for IGF I and IGF II (Rechler 1985; Verspohl et al. 1988). They belong to the insulin receptor family of receptor tyrosine kinases (Ullrich and Schlessinger 1990; Schlessinger and Ullrich 1992; Fantl et al. 1993). Although insulin and IGF bind with high affinity to

their own specific receptors, each can also bind to the heterologous receptor with reduced binding affinity.

With recombinant DNA technology insulin analogues with modified amino acid sequence can be produced which may bind differently to the insulin and IGF receptor resulting in different biological activities (Drejer 1992; Schäffer 1993). It has been speculated whether the unexpected carcinogenic effect of an insulin analogue (Dideriksen et al. 1992) is related to the mitogenic effect in mouse NIH 3T3 fibroblasts which express IGF receptors but not insulin receptors (Gammeltoft and Drejer 1991). Biosynthesis of 10 kDa and 7.5 kDa insulin-like growth factor II in a human rhabdomyosarcoma cell line has been described by Nielsen et al. (1993).

For new insulin analogues the kinetics of association and dissociation to the receptors should be studied and *in vivo* metabolic activity should be compared with insulin and IGF I.

The *in vivo* metabolic action of insulin-like growth factor I can be compared with insulin in adult rats using the following methods (Schmitz et al. 1991):

Dose-dependence and time-dependence of blood sugar decrease after intravenous injection,

antilipolytic effect (decrease of non-esterified fatty acids) after i.v. injection,

stimulation of glucose disposal during euglycemic clamping after intravenous infusion.

Furthermore, all other assays described for insulin can be applied.

Gazzano-Santoro et al. (1998) described a cell-based potency assay for insulin-like growth factor-1.

PROCEDURE

Cells from the human cell line HU-3, established from the bone marrow of a patient with acute megakaryoblastic leukemia, are adapted to grow in the presence of human thrombopoietin. Removal of thrombopoietin results in decreased proliferation and rapid loss of viability. Cells are cultured in RPMI-1 640 medium with 2% heat-inactivated human serum, 2 mM glutamine, 10 mM HEPES (pH 7.2) and 5 ng/ml thrombopoietin in culture flasks. They are grown in suspension at 37 °C in a humidified 5% CO_2 incubator and are routinely subcultured every two or three days when densities reach $0.8–1.5 \times 10^6$ cells/ml.

The cell growth assay is performed under serum-free conditions in Assay Medium consisting of RPMI-1 640 supplemented with 0.1% BSA, 10 mM HEPES (pH 7.2) and 50 µg/ml gentamycin. Cells are washed twice in the Assay Medium and resuspended at a density of 0.25×10^6 cells/ml. In a typical assay, 100 µl of a cell suspension (25 000 cells/well) and 100 µl of IGF-1 at varying concentrations are added to flat bottomed 96-well tissue culture plates at 37 °C and 5% CO_2 and cultured for 2 days. Forty microliters of Alamar Blue™ (undiluted) are then added and the incubation continued for 7–24 h. The plates are allowed to cool to room temperature for 10 min on a shaker and the fluorescence is read using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm.

EVALUATION

Results, expressed as relative fluorescence units, are plotted against IGF-1 concentrations using a 4-parameter curve-fitting program. Test compounds are compared with the standard.

MODIFICATIONS OF THE METHOD

Boge et al. (1994) described an enzyme immunoreceptor assay for the quantitation of insulin-like growth factor-1 and insulin receptors in bovine muscle tissue.

Hodgson et al. (1995) tested mutations at positions 11 and 60 of insulin-like growth factor-1 using (a) quantification of affinities for the human insulin receptor overexpressed on NIH 3T3 cells, (b) quantification of affinities for the type 1 IGF receptor via competition for binding to a monolayer of MDA-MB-231 cells, (c) promotion of the *in vitro* mitogenesis of growth-arrested MCF-7 cells in the presence of 17-β-oestradiol, and (d) a competition assay for binding to IGF-binding proteins secreted by MCF-7 cells.

In order to investigate the influence of insulin-like growth factor-1 and IGF-binding protein-1 on wound healing, Lee et al. (1996) measured the contraction of collagen gels with embedded fibroblasts.

Ernst and White (1996) studied the hormonal regulation of IGF-binding protein-2 expression in C_2C_{12} myoblasts.

Frystyk and Baxter (1998) described a competitive assay for rat insulin-like growth factor (IGF)-binding protein-3 (rIGFBP-3) based on the ability of IGFBP-3 to form a ternary complex with the acid labile subunit (ALS) in the presence of IGF. Human ALS was bound to test tubes pre-coated with anti-human ALS antibody. The assay depends on a competition between a covalent complex of ^{125}I-hIGF-I and hIGFBP-3, added as tracer, and hIGFBP-3 or rIGFBP-3 in standards or test samples, for binding to the immobilized hALS. Purified natural hIGFBP-3 served as standard.

Damon et al. (1997) used the C_2 skeletal myogenic cell line to characterize an insulin-like growth factor-binding protein, named Mac25/IGFBP-7.

To explore the possible relationship between insulin-like growth factor I and diabetic retinopathy, Naruse et al. (1996) examined the effects of glucose on IGF-I stimulated thymidine incorporation into DNA and IGF-I binding in cultured bovine retinal pericytes.

Jonsson et al. (1997) used a fluorometric proliferation assay with AlamarBlue to study the proliferative capacity of isolated human osteoblasts which were dose-dependently stimulated by IGF-I.

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K.4.0.5 Amylin

K.4.0.5.1 General considerations

PURPOSE AND RATIONALE

Amylin, also named islet amyloid polypeptide, is a pancreatic islet peptide consisting of 37 amino acids with a role in the maintenance of glucose homeostasis. The peptide is predominantly present in the β-cells of the pancreas and to a lesser extent in the gastrointestinal tract and in the nervous system, where amylin mRNA is also present along with specific binding sites.

Amylin has structural and functional relationships to two other messenger proteins, calcitonin and calcitonin gene-related peptide (Rink et al. 1993; Pittner et al. 1994; van Rossum et al. 1997; Wimalawansa 1996).

Amylin inhibits food intake in rodents when given centrally as well as peripherally (Morley et al. 1997; Lutz et al. 1998). Bhavsar et al. (1998) found a synergy between amylin and cholecystokinin for inhibition of food intake in mice.

Amylin reduces insulin-stimulated incorporation of glucose into glycogen in skeletal muscle. Young et al. (1992) found that increasing concentrations of amylin progressively depressed the maximal insulin-stimulated radioglucose incorporation into soleus muscle glycogen.

Castle et al. (1998) reported an inhibition of 3-O-methyl-D-glucose transport in perfused rat hindlimb muscle under hyperinsulinemic conditions.

Bryer-Ash et al. (1995) found that amylin-mediated reduction in insulin sensitivity corresponds to reduced insulin receptor kinase activity in the rat *in vivo*.

Transgenic mice expressing human islet amyloid polypeptide develop diabetes mellitus by 8 weeks of age, which is associated with selective β-cell death and impaired insulin secretion (Janson et al. 1996).

Mulder et al. (2000) showed that amylin-deficient mice develop a more severe form of alloxan-induced diabetes.

In single β-cells exhibiting normal glucose sensing, amylin causes membrane hyperpolarization, increases net outward current and reductions in insulin secretion. In contrast, in cells with abnormal glucose sensing (e.g., from *db/db* diabetic mice) amylin has no effect on electrical activity or secretion (Wagoner et al. 1993).

Amylin suppressed the cAMP generation induced by glucagon-like peptide 1 (GLP-1) in RINm5F cells dose-dependently (Göke et al. 1993).

Amylin, also named human islet amyloid polypeptide (hIAPP) according to van Hulst et al. (1997), may be implicated in the pathogenesis of pancreatic islet amyloid formation and type 2 diabetes mellitus (Wimalawansa 1997). Hulst et al. (1997) used transgenic mice for the study of (patho)physiological roles of hIAPP *in vivo*. Leckstrom et al. (1997) studied plasma levels and immunoreactivity of insulin and islet amyloid polypeptide in *Psammomys obesus* (sand rat) under low-energy and high-energy diet.

Amylin given peripherally or centrally inhibits acid gastric secretion in a dose dependent manner and has a protective effect against indomethacin- or ethanol-induced ulcers only when injected centrally. Subcutaneous or central injection of amylin produces a dose-dependent inhibition of gastric emptying, which may contribute to the activity of amylin in the regulation of carbohydrate absorption. In addition, amylin inhibits food intake both when injected peripherally or centrally. Amylin is considered to take part in the rapid endocrine response during digestion to maintain euglycemia (Guidobono 1998).

Amylin slows the rate of gastric emptying in spontaneously diabetic BB/Wistar rats (Macdonald 1997) and inhibits gastric acid secretion in rats (Rossowski et al. 1997).

Guidobono et al. (1997) reported a cytoprotective effect of amylin against indomethacin- and ethanol-induced ulcers when given intracerebroventricularly, but not when given subcutaneously.

Clementi et al. (1997) found a protective, dose-dependent effect of amylin against reserpine- and serotonin-induced gastric damage in rats, however, no anti-ulcer effect after pylorus ligation.

The search for a superior compound with the biological actions of human amylin resulted in the identification of [Pro^{25,28,29}]human amylin, assigned the USAN name pramlintide. Young et al. (1996) compared this compound with human and rat amylin in various pharmacological tests, such as receptor assays (binding to rat nucleus membranes, amylin receptors; binding to membranes from SK-N-MC cells, CGRP receptors; binding to membranes from T46D cells, calcitonin receptors), gastric emptying in rats, plasma glucose after oral glucose gavage. When studying glycogen metabolism in the isolated soleus muscle of rats, pramlintide was slightly more potent than human amylin, but not different from rat amylin in inhibiting insulin-stimulated incorporation of [U-^{14}C]glucose into muscle glycogen. After intravenous infusion of amylin or pramlintide, a dose-dependent increase of plasma glucose, plasma lactate and ionized calcium was found. Mean arterial blood pressure decreased with higher doses.

Clementi et al. (1995) found an anti-inflammatory activity of amylin in mouse ear edema induced by Croton oil and acetic acid-induced peritonitis in the rat, but not in serotonin-induced rat paw edema and plasma protein extravasation induced by dextran in rat skin.

Bell et al. (1995), Bell and McDermont (1995) reported that amylin has an hypertrophic effect in rat ventricular cardiomyocytes and exerts the contractile response via $CGRP_1$-preferring receptors.

In anesthetized rats, amylin increased after intravenous infusion urine flow, sodium excretion, glomerular filtration rate and renal plasma flow (Vine et al. 1998).

Clementi et al. (1996) reported that intracerebroventricularly injected amylin induced in rats a dose-dependent decrease of locomotor activity without affecting grooming and sniffing.

Amylin dose-dependently stimulated cell proliferation of human osteoblast like (hOB) cells and increased osteocalcin production (Villa et al. 1997). Cornish et al. (1998) found in adult male mice after daily subcutaneous injection of amylin for 4 weeks an increase of histomorphometric indices of bone formation and a reduction of bone resorption.

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K.4.0.5.2 Receptor binding of amylin

PURPOSE AND RATIONALE

Binding sites with high affinity for amylin are present in several brain regions, with the nucleus accumbens and surrounding tissue containing more than twice as many banding sites as any other regions (Beaumont et al. 1993).

PROCEDURE

Membranes are prepared from male Sprague-Dawley rats (150–200 g). Following decapitation, the basal forebrain regions (nucleus accumbens) are removed to phosphate buffered saline (PBS), pH 7.4 at 4 °C. The tissues are weighted, then placed in 10 ml/g tissue of ice-cold 20 mM HEPES buffer, pH 7.4 and homogenized with a Polytron (10 s at setting 4). An addi-tional 30 ml of cold HEPES is added, and the homogenate centrifuged at 48 000 *g* for 15 min. After discarding the supernatant fluid, membrane pellets are resuspended by homogenization in 40 ml of fresh HEPES buffer and centrifuged as before. Membranes are washed again by homogenization in buffer and centrifugation. The final membrane pellet is resuspended in a volume of 20 mM HEPES containing 0.2 mM PMSF added immediately before use from a stock 0.2 M solution in ethanol. A volume of buffer is used to yield a concentration of about 80 mg original tissue/ml. Membranes are kept frozen at –80 °C until use.

Membranes from 4 mg of original wet weight of tissue are incubated with ^{125}I-BH-amylin (rat amylin, BH-labeled at the amino-terminal lysine, (Amersham Corp.) in 20 mM HEPES buffer, containing 0.5 mg/ml bacitracin, 0.5 mg/ml bovine serum albumin, and 0.2 mM phenylmethylsulfonyl fluoride, for 60 min at 23 °C. Incubations are carried out in duplicate tubes and are started by addition of membranes. Incubations are terminated by filtration through glass fiber filters that have been presoaked in 0.3% polyethylene-imine, followed by washing with 15 ml of cold phosphate-buffered saline.

EVALUATION

Competition curves are generated by measuring binding of 13 pM ^{125}I-BH-amylin in the presence of 10^{-11} to 10^{-6} unlabeled peptide. Data are fitted to a four-parameter logistic equation to derive half-maximal inhibitory concentrations (IC_{50} values) and slope factors (Hill coefficients).

MODIFICATIONS OF THE METHOD

Sheriff et al. (1992) characterized amylin binding sites in the human hepatoblastoma cell line, HepG2.

Perry et al. (1997) studied amylin and calcitonin receptor binding in the mouse α-thyroid-stimulating hormone thyrotroph cell line.

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K.5 Blood glucose lowering activity of antidiabetic drugs

K.5.0.1 Blood glucose lowering activity in rabbits

PURPOSE AND RATIONALE

The rabbit has been used since many years for standardization of insulin (see K.3.1.1.). Therefore, it has been chosen as primary screening model for screening of blood glucose lowering compounds as well as for establishing time-response curves and relative activities (Bänder et al. 1969; Geisen 1988).

PROCEDURE

Groups of 4–5 mixed breed rabbits (e.g. Hoe:BASK, SPFWiga) of either sex weighing 3.0–4.5 kg are used. For insulin evaluation, food is withheld overnight. For evaluation of sulfonylureas and other blood glucose lowering agents the animals are on a normal diet (e.g. Era® mixed feed 8 300) prior to the experiment. The animals are gently placed into special restraining boxes allowing free access to the rabbit's ears.

Oral blood glucose lowering substances are applied by gavage in 1 ml/kg of 0.4% starch suspension or intravenously in solution. Several doses are given to different groups. One control group receives the vehicle only. By puncture of the ear veins, blood is withdrawn immediately before and 1, 2, 3, 4, 5, 24, 48, and 72 h after treatment. For time-response curves values are also measured after 8, 12, 16, and 20 h. Blood glucose is determined in 10 µl blood samples with the hexokinase enzyme method (Glucoquant® test kit).

MODIFICATIONS OF THE METHOD

For special purposes the effect of blood sugar lowering agents is studied in glucose loaded animals. Rabbits of either sex weighing 3.0–4.5 kg are treated either once (0.5 h after test compound) or twice (0.5 and 2.5 h after test compound) orally with 2 g glucose/kg body weight in 50% solution.

EVALUATION

Average blood sugar values are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Mean effects at a time interval are calculated using the trapezoidal rule. The values of the experimental group are compared statistically with the t-test or the WILCOXON test for each time interval with those of the control group. Differences between several treated groups and the control group are tested using a simultaneous comparison according to Dunnett or Nemenyi/Dunnett (1966). Dose dependencies and relative activities are determined by means of linear regression analysis after Fieller (1944) and Sidak (1967). All data for statistical comparisons have to be tested for homogeneity of variances according to Levene (1960) and for normal distribution according to Shapiro and Wilk (Miller 1965). In the case of regression analyses, the lines are additionally tested for parallelism according to Tuckey (1966) and for linearity according to Scheffé (1959). The level of significance for all procedures is chosen as 5%.

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K.5.0.2 Blood glucose lowering activity in rats

PURPOSE AND RATIONALE

Rats are used for screening as well as for quantitative evaluation of blood glucose lowering agents.

PROCEDURE

Male Wistar rats (e.g. Hoe:WISKf, SPF 71) weighing 180–240 g are kept on standard diet (e.g. Altromin® 1 324). Groups of 4–7 non-fasted animals are treated orally or intraperitoneally with various doses of the test compounds suspended in 0.4% starch suspension. One control group receives the vehicle only. Blood is withdrawn from the tip of the tail immediately before, and 1, 2, 3, 5, and 24 h after administration of the test compound. Blood glucose is determined in 10 µl blood samples with the hexokinase enzyme method (Glucoquant® test kit).

EVALUATION

Average blood sugar values are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Mean effects over a time period are calculated using the trapezoidal rule. Statistical evaluation is performed as described for tests in rabbits.

MODIFICATIONS OF THE METHOD

Studies in glucose loaded rats

For special purposes the effect of blood sugar lowering agents is studied in glucose loaded animals. One g glucose/kg body weight is given in a 50% solution either orally 5 min after oral administration or subcutaneously 5 min after intraperitoneal administration of the test compound.

Studies in streptozotocin-diabetic rats

Male Wistar rats (e.g. Hoe:WISKf, SPF 71 strain) weighing 170–220 g are kept on standard diet (e.g. Altromin® 1 324). Ten–fourteen days prior to the study they are injected with 60 mg/kg streptozotocin (Calbiochem) intravenously. Blood sugar levels rise from 5.5–6.0 mmol/l up to 25.0–28.0 mmol/l and glucosuria occurs. Plasma insulin levels fall below 4 µU/ml.

Compounds which release insulin from pancreatic islets as sole hypoglycemic activity are not effective in rats with severe streptozotocin induced diabetes.

Studies in guinea pigs

Instead of rats male guinea pigs (e.g., Pirbright white, Hoe:DHPK, SPF Lac) weighing 250–380 g can be used. Blood is withdrawn by puncture of ear veins before, and 1, 3, and 5 h after administration of the test compound or the vehicle. Blood sugar determinations and statistical evaluations are performed as described for rabbits and rats.

Studies in genetically diabetic animals

Genetically obese and diabetic yellow KK mice have been used by Sohda et al. (1990) for evaluation of hypoglycemic activity of potential antidiabetic drugs.

Gill and Yen (1991) studied the effect of ciglitazone in obese-diabetic viable yellow mice (VY/Wfl-A^{vy}/a).

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K.5.0.3 Blood glucose lowering activity in dogs

PURPOSE AND RATIONALE

Besides experiments in rats and rabbits studies in dogs are necessary to predict the effect of a new compound in man due to differences in species related metabolism.

PROCEDURE

Male Beagle dogs weighing 15–20 kg are kept on standard diet (e.g. Erka mixed feed 8 500). Food is withdrawn 18 h prior to the administration of the test compound which is given either orally or intravenously in various doses. Control animals receive the vehicle only. Blood is collected at different time intervals up to 48 h. Blood glucose is determined with the hexokinase enzyme method (Glucoquant® test kit) and plasma insulin with an immunological method (Riagnost®-kit).

EVALUATION

Average blood sugar values are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Mean effects over a time period are calculated using the trapezoidal rule. Similarly, plasma insulin levels are plotted versus time and compared with control values. Statistical evaluation is performed as described for tests in rabbits.

MODIFICATIONS OF THE METHOD

Studies in pancreatectomized dogs

The surgical technique of pancreatectomy in dogs is described in Sect. K.1.0.1. The animals are pancreatectomized up to 2–3 years prior to the study. They are kept on dry feed (Vipromix®) together with 2–3 g pancreatic enzymes (Viveral®). Insulin is substituted with a single daily subcutaneous dose of 32 IU Insulin Ultratard HM®. For substitution of vitamin D an intramuscular dose of 1 ml Vigantol® is given every 3 months.

On the day before the study, the animals receive 32 IU of the shorter acting Basal-H® insulin. This insulin is administered at the same time when food and test compound are given in the morning. The test drug

is applied as oral suspension in tap water. Blood glucose is determined before and up to 6 h after treatment in hourly intervals. Control animals receive tap water only.

Studies in alloxan-diabetic dogs

Chemical diabetes can be induced by a single intravenous dose of 60 mg/kg alloxan. Afterwards, the animals receive infusions of 1 000 ml 5% glucose together with 10 IU Regular® Insulin via a jugular vein catheter daily during one week and canned food ad libitum. Thereafter, a single dose of 28 IU Insulin Ultratard HM® is given daily and the animals are fed commercial diet (Altromin® pellets). On the day before the study, the dogs receive 28 IU of the shorter acting Basal-H® insulin. This insulin is given at the same time as food and test compound in the morning. The test drug is applied as oral suspension in tap water. Blood glucose is determined before and up to 6 h after treatment in hourly intervals. Control animals receive tap water only.

Continuous blood glucose monitoring

A device for continuous blood glucose monitoring and infusion in freely mobile dogs was described by Geisen et al. (1981).

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K.5.0.4 Euglycemic clamp technique

PURPOSE AND RATIONALE

The euglycemic glucose clamp technique has provided a useful method of quantifying *in vivo* insulin sensitivity in humans (DeFronzo et al. 1979). In this technique a variable glucose infusion is delivered to maintain euglycemia during insulin infusion. Whole-body tissue sensitivity to insulin, as determined by net glucose uptake, can be quantitated under conditions of near steady state glucose and insulin levels. Kraegen et al. (1983, 1985) developed the euglycemic glucose clamp technique for use in the intact conscious rat.

PROCEDURE

Male Wistar rats weighing 150–200 g are fasted overnight and anesthetized with pentobarbital (40 mg/kg, i.p.). Catheters are inserted into a jugular vein and a femoral vein for blood collections and insulin and glucose infusion, respectively. To evaluate the insulin action under physiological hyperinsulinemia (steady state plasma insulin concentration during the clamp test around 100 μU/dl), and maximal hyperinsulinemia (under which maximal insulin action may appear) two insulin infusion rates, 6 and 30 mU/kg/min, are used. The blood glucose concentrations are determined from samples collected at 5-min intervals during the 90-min clamp test. The glucose infusion rate is adjusted so as to maintain the blood glucose at its basal level during the clamp test. The final glucose infusion rate is calculated from the amount of glucose infused for the last 30 min (from 60 to 90 min after start of the clamp) in which the blood glucose levels are in a steady state. The glucose metabolic clearance rate is obtained by dividing the glucose infusion rate by the steady state blood glucose concentration. The steady state plasma insulin concentration is calculated from the insulin concentrations at 60 and 90 min after the start of the clamp. At the start and end of the euglycemic clamp test, free fatty acid concentration is also determined and the free fatty acid suppression rate is calculated.

EVALUATION

All values are analyzed by one-way ANOVA.

When the steady state plasma insulin is maintained at submaximal concentration by the euglycemic clamp technique, the glucose infusion rate and glucose metabolic clearance rate value are considered to reflect the state of receptor binding levels in the peripheral tissue as an index for insulin sensitivity. Under maximal hyperinsulinemia these values are thought to reflect the state of the enzymes and glucose transport system activated after the binding to receptors, indicating mainly insulin responsiveness.

MODIFICATIONS OF THE METHOD

Burnol et al. (1983) used the euglycemic insulin clamp technique coupled with isotopic measurement of glucose turnover to quantify insulin sensitivity in the anesthetized rat.

Bryer-Ash et al. (1995) used this technique to demonstrate reduction of insulin sensitivity by amylin corresponding to reduced insulin receptor kinase activity.

The effects of counterregulatory hormones on insulin-induced glucose utilization by individual tissues in rats, using the euglycemic hyperinsulinemic clamp technique combined with an injection of 2-[1-^{3}H]-deoxyglucose, were studied by Marfaing et al. (1991).

Lang (1992) determined the insulin-mediated glucose uptake in normal and streptozotocin-diabetic rats using the euglycemic-hyperinsulinemic clamp technique.

Hirshman and Horton (1990) reported increased insulin sensitivity and responsiveness in peripheral tissues of the rat after glyburide as determined by the glucose clamp technique.

Lee et al. (1994) studied the metabolic effects of troglitazone on fructose-induced insulin resistance with the euglycemic hyperinsulinemic clamp technique in rats.

Tominaga et al. (1992, 1993) studied the influence of insulin antibodies in anesthetized rats and of thiazolidinediones on hepatic insulin resistance in streptozotocin-induced diabetic rats by the glucose clamp technique.

Gelardi et al. (1991) used the hyperinsulinemic-euglycemic clamp technique to evaluate the insulin sensitivity in the obese offspring of streptozotocin-induced mildly hyperglycemic rats.

Hulman et al. (1993) studied insulin resistance in the conscious spontaneously hypertensive rat with the euglycemic hyperinsulinemic clamp technique.

Cheung and Bryer-Ash (1994) described a modified method for the performance of glucose insulin clamp studies in conscious rats under local anesthesia.

Burvin et al. (1994) developed a modification of the euglycemic insulin clamp technique and used it to repeatedly assess *in vivo* insulin effects in awake streptozotocin-induced diabetic rats.

Xie et al. (1996) described a modified euglycemic clamp technique in **cats**.

Finegood et al. (1987) estimated endogenous glucose production during hyperinsulinemic-euglycemic glucose clamps using unlabeled and labeled glucose infusates in **dogs**.

Xie et al. (1996) described an insulin sensitivity test using a modified euglycemic clamp in **cats** and rats. This test uses the amount of glucose required to be infused to maintain euglycemia over a 30-min period in rats and 60 min in cats following a bolus administration of insulin as the index of insulin sensitivity. Glucose levels are determined a short intervals and variable glucose infusion is used to hold glucose levels within a few percentage points of the basal pre-test glucose level. A new blood sampling technique is described that allows each insulin sensitivity test to be carried out using a total of only 0.5 ml of blood.

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K.5.0.5 Effects of insulin sensitizer drugs

PURPOSE AND RATIONALE

Insulin sensitizer drugs are reported to improve symptoms in patients with established NIDDM (non-insulin dependent diabetes mellitus) (Colca 1995; Kuehnle 1996). In contrast to sulfonylureas, these compounds do not lower blood glucose in normal animals. Various animal models resembling type II diabetes are used for evaluation. Details of these models are described in the respective chapters. *In vitro* techniques showed an increased glucose uptake of into muscle tissue and into adipocytes.

PROCEDURES

In vivo studies

Chang et al. (1983) studied ciglitazone in *ob/ob* and *db/db* mice, diabetic Chinese hamsters, and normal and streptozotocin-diabetic rats.

Fujita et al. (1983) investigated the effects of ciglitazone in obese-diabetic yellow *KK* (*KK-A*^{y}) mice and obese Zucker-fatty rats.

Diani et al. (1984) treated C5BL/6J-*ob/ob* and C57BL/KsJ-*db/db* mice for several weeks with ciglita-zone and studied the morphological effects on pancreatic islets.

Fujiwara et al. (1988) performed studies in *KK* and *ob/ob* mice and Zucker fatty rats.

Moreover, Fujiwara et al. (1991) studied the effects of CS-045 on glycemic control and pancreatic islet structure at a late stage of the diabetes syndrome in C57BL/KsJ-*db/db* mice.

Ikeda et al. (1990), Sohda et al. (1990) used insulin resistant animals (yellow *KK* mice, Zucker fatty rats, and obese Beagle dogs with moderate insulin resistance).

Gill and Yen (1991) studied the effects on endogenous plasma islet amyloid polypeptide and insulin sensitivity in obese-diabetic viable yellow mice.

Hofmann et al. (1991, 1992) treated insulin resistant KKA^y mice.

Stevenson et al. (1991) studied the effects of englitazone in nondiabetic rats and found no overt hypoglycemia but an enhancement of insulin action.

Kobayashi et al. (1992) found an increase of insulin sensitivity by activating insulin receptor kinase in genetically obese Wistar fatty rats treated with various doses of pioglitazone.

Sugiyma et al. (1992) found a reduction of glucose intolerance and hypersecretion of insulin in Wistar fatty rats after treatment with pioglitazone for 10 days.

Tominaga et al. (1993) used the glucose clamp-technique in streptozotocin-induced diabetic rats.

Yoshioka et al. (1993) found antihypertensive effects in obese Zucker rats.

Lee et al. (1994) studied the metabolic effects on fructose-induced insulin resistance in rats.

Apweiler et al. (1995) administered BM 13.09143 to lean and obese Zucker rats and performed hyperinsulinemic-euglycemic clamp studies in these animals.

Fujiwara et al. (1995) found a suppression of hepatic gluconeogenesis in long-term troglitazone treated diabetic KK and C57BL/ksJ-*db/db* mice.

Lee and Olefsky (1995) studied the effects of troglitazone in normal rats with the euglycemic glucose clamp technique.

In vitro studies

Kirsch et al. (1984) found a reversal of cAMP-induced post-insulin receptor resistance in rat adipocytes *in vitro*.

Ciaraldi et al. (1990) performed *in vitro* studies using cultured hepatoma cells (Hep G2) and muscle cells (BC3H-1) and found an increased glycogen synthase I activity in both cell types.

Murano et al. (1994) found a stimulation of fructose-2,6-bisphosphate production in rat hepatocytes.

Kellerer et al. (1994) reported the prevention of glucose-induced insulin resistance of insulin receptor in rat-1 fibroblasts.

Bader et al. (1993) found an increased [^{32}P]incorporation in the 95 kD β-subunit of the insulin receptor and an increased phosphorylation of the synthetic substrate Poly[GluNa4 : 1Tyr] in receptors isolated from skeletal muscle of obese Zucker rats treated with CS 045.

Teboul et al. (1995) found that thiazolidinediones convert myogenic cells (C_2C_{12}N myoblasts, a subclone of the C_2C_{12} cell line) into adipose-like cells. Thiazolidinediones or fatty acids prevented the expression of myogenin, α-actin, and creatine kinase and abolished the formation of multinucleated myotubes. In parallel, these treatments induced the expression of a typical adipose differentiation program including acquisition of adipocyte morphology and activation of adipose-related genes.

Tafuri (1996) reported that troglitazone enhanced the rate and percent differentiation of fibroblasts to adipocytes. Basal glucose transport and synthesis of GLUT1 transporter messenger RNA were increased.

Stevenson et al. (1990) examined the effects of racemic englitazone (CP 68 722) in adipocytes and soleus muscles from *ob/ob* mice and in 3T3-L1 adipocytes. Administration of the drug in various doses lowered plasma glucose and insulin dose-dependently without producing frank hypoglycemia in either diabetic or nondiabetic animals. Basal and insulin-stimulated lipo-

genesis were enhanced in adipocytes from *ob/ob* mice. Glycogenesis and basal glucose oxidation in isolated soleus muscles were stimulated.

Kreutter et al. (1990) used 3T3-L1 adipocytes and found a stimulation of 2-deoxyglucose uptake.

Masuda et al. (1995) found an insulinotropic mechanism distinct from glibenclamide in isolated rat pancreatic islets and HIT cells.

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K.5.0.6 Effects of thiazolidinediones on peroxisome proliferator-activated receptor-γ

PURPOSE AND RATIONALE

Peroxisome proliferator-activated receptors (PPARs) compose a subfamily of the nuclear hormone receptors. Three distinct PPARs, termed α, δ, and γ, each encoded by a separate gene and showing a distinct tissue distribution pattern, have been described (Keller and Wahli 1993; Green 1995; Devchand et al. 1996; Lemberger et al. 1996; Schoonjans et al. 1996a,b, 1997). Ligands that induce the transcriptional activity of PPARα and γ have been identified (Forman et al. 1995; Devchand et al. 1996; Lehmann 1995). PPARγ is a central regulator of adipocyte gene expression and differentiation (Tortonoz et al. 1995; Brun et al. 1996; Wu 1998). Thiazolidinedione derivatives which are antidiabetic agents are potent and selective activators of PPARγ (Young et al. 1997; Murakami et al. 1998; Reginato et al. 1998; Ribon et al. 1998). Berger et al. (1996) found a correlation of antidiabetic actions of thiazolidinediones in *db/db* mice with the conformational change in peroxisomal proliferator-activated receptor-γ: Murphy and Holder (2000) suggested a therapeutic potential of PPARγ agonists in the treatment of inflammatory diseases and certain cancers.

Steppan et al. (2001), Berger (2001) showed that adipocytes secrete a signaling molecule which they called resistin (for resistance to insulin). Circulating resistin levels in mice are decreased by thiazolidindiones and are increased in diet-induced and genetic forms of obesity.

PROCEDURE

Plasmids

The pSG5-haPPARγ1 expression construct is generated by inserting the hamster PPARγ complementary DNA into the EcoRI site of the pSG5 expression vector (Strategene, La Jolla, CA). As reporter construct, pPPRE-chloramphenicol acetyltransferase (CAT) is used containing two copies of the peroxisomal proliferator response element from the enhancer region of the murine acyl coenzyme A-oxidase gene adjacent to the glutathione-S-transferase minimal promotor and the CAT gene.

Cell culture and transfections

COS-1 cells are seeded at 2.1×10^5 cells per dish in 35-mm dishes (for transactivation assays) and 3×10^6 cells/dish in 150-mm dishes (for binding assays) in DMEM (high glucose) containing 10% charcoal-stripped fetal calf serum, nonessential amino acids, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate at 37 °C in a humidified atmosphere of 10% CO_2. After 24 h, transfections are performed with Lipofectamine (Life Technologies, Gaithersburg, MD). For transactivation experiments, transfection mixes are used containing 1 µg receptor expression vector, and 1 µg pCH110 (Pharmacia, Piscataway, NJ) as an internal control and, for binding studies 20 µg receptor expression vector.

Binding assay

Transfected cells are grown for 48 h after transfection with the receptor expression vector. Receptor preparation is performed according to Tilley et al. (1989). Cell lysates containing receptor are prepared in TEGM (10 mM Tris-HCl, pH 7.2; 1 mM EDTA; 10% glycerol; 7 µl/100 ml β-mercaptoethanol; 10 mM Na molybdate; 1 mM dithiothreitol; 5 µg/ml aprotinin; 2 µg/ml leupeptin; 2 mg/ml benzamide; and 0.5 mM phenylmethylsulfonylfluoride). Plates are placed on ice, rinsed with TEG (10 mM Tris-HCl, pH 7.2; 50 mM EDTA; and 10% glycerol), and scraped into 0.5 ml TEGM. The material is pooled, frozen in liquid nitrogen to lyse the cells, and thawed on ice. The lysate is centrifuged at 22 000 *g* for 20 min at 4 °C to remove the debris and stored frozen (–80 °C) until use. For each assay, an aliquot of receptor-containing lysate (0.1–0.25 mg protein) is incubated with 10 nM ditritiated AD-5 075 (21 Ci/mmol) with or without test compound for ~16 h at 4 °C in TEGM (300 µl final volume). Unbound ligand is removed by incubation on ice for ~10 min after the addition of 200 µl dextran/gelatin-coated charcoal. After centrifugation at 3 000 rpm for 10 min at 4 °C, 200-µl aliquots of supernate are counted in an liquid scintillation counter.

Transactivation assay

After transfection, cells are incubated for 48 h in culture medium with or without increasing concentrations of test compounds. Cell lysates are produced using reporter lysis buffer (Promega Corp., Madison, WI). CAT activity is determined using radiolabeled butyryl CoA as substrate in a diffusion based assay. β-Galactosidase activity is determined according to Hollons and Yoshimura (1989).

Protease digestion assay

The protease digestion assay is performed according to the method of Allan at al (1992) with minor modifications. The plasmid pSG5-haPPARγ1 is used to synthesize ^{35}S-radiolabeled PPARγ1 in a coupled transcription/translation system. The transcription/translation reactions are subsequently aliquoted into 22.5-µl vol-

umes, and 2.5 µl PBS with or without a thiazolidinethione are added. The mixtures are incubated for 20 min at 25 °C, separated into 4.5-µl aliquots, and 0.5 µl distilled water or distilled water-solubilized trypsin are added. The protein digestions are allowed to proceed for 10 min at 25 °C, then terminated by the addition of 20 µl denaturing gel loading buffer and boiling for 5 min. The products of the digestion are separated by electrophoresis through a 1.5-mm 12% polyacrylamide-SDS gel. After electrophoresis, the gels are fixed in 10% acetic acid-40% methanol for 30 min, treated in EN^{3}HANCE for an additional 30 min. and dried under vacuum for 2 h at 80 °C. Autoradiography is then performed to visualize the radiolabeled digestion products.

EVALUATION

Binding assays

For binding assays, competition curves are generated by incubation of 10 nM [^{3}H]AD-5075 with hamster PPARg1 produced by transient transfection of COS-1. The percentage of ligand bound after incubation in the presence of the indicated concentration of each unlabeled compound for 16 h is plotted.

Activation assays

For activation assays of PPARg in COS-1 cells transiently cotransfected with pSG5-haPPARg1 and pPPRE-CAT, normalized CAT activity is plotted after incubation in the presence of the indicated concentration of each compound for 48 h.

Protease digestion assay

For evaluation of the protease digestion assay, the partially protease-resistant conformation product of PPARg is visualized by autoradiography on SDS-PAGE after incubation with the thiazolidinedione and increasing concentrations of trypsin.

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K.6 Activity in isolated organs, cells, and membranes

K.6.1 Effects on pancreas

K.6.1.1 Perfusion of isolated rat pancreas

PURPOSE AND RATIONALE

The *in vitro* perfusion of the isolated rat pancreas as described by Anderson and Long (1947) and by Grodsky et al. (1963, 1984; Muñoz et al. 1995) offers the advantage to study the influence of carbohydrates, hormones and drugs such as sulfonylureas not only on insulin but also on glucagon and somatostatin secretion without interference of secondary effects resulting from changes in hepatic, pituitary or adrenal functions.

PROCEDURE

Male Wistar rats (e.g. Hoe:WISKf, SPF 71-strain) weighing 200–250 g serve as donors. Prior to the experiment, the animals have free access to food and water. The pancreas is removed under pentobarbital (50 mg/kg i.p.) anesthesia. The mesenteric artery is doubly ligated and cut, and the entire intestine below the duodenum is separated and removed from the rat to simplify the exposure. The esophagus is ligated as high as possible and cut above the ligature. A loose ligature is placed, but not tied, around the entire gastrohepatic ligament. The aorta is cautiously exposed through the crura of the diaphragm well above the point of origin of the celiac axis. The ligature around the gastrohepatic ligament is then tied as tightly as possible, and the ligament cut above the ligature. The aorta is cut between the double clamps and below the lower clamp, and the preparation is lifted out from the abdomen. The clamps are removed from the aorta which is slit open on the side opposite to the origin of the celiac axis, revealing the opening of the latter. The arterial cannula is inserted and tied in place. An opening is made close to the ligature, as near as the end of the portal vein as possible.

Circulation through the preparation is initiated and fluid is observed to flow from the slit in the portal vein. After a minute or two, the flow is stopped and a cannula is inserted into the portal vein and tied in place. Flow is then resumed at a perfusion pressure of about 100 mm Hg. Carbogenated Krebs-Ringer bicarbonate buffer with 2% bovine albumin and 5.5 mmol/l glucose is used as perfusion medium at a temperature of 37.5 °C with a perfusion rate of 1.75 ml/min. The perfusate is collected every minute for 30 min. After 5 min perfusion with 5.5 mM glucose, the test compound is added until the 15th min (concentrations between 0.05 and 0.5 mM for highly active substances), followed by perfusion with 5.5 mM glucose and finally with 16.6 mM glucose.

The perfusate medium samples are stored at –20 °C until further processing.

EVALUATION

The hormones: insulin, glucagon and somatostatin are determined radioimmunologically: insulin with the RIAgnost®-kit and rat insulin as standard; glucagon with a rabbit antibody, ^{125}I-glucagon as tracer, and polyethylene glycol as precipitant; somatostatin with a rabbit antibody, ^{125}I-tyrosyl-somatostatin as tracer and charcoal dextran for separation of free and bound hormone. At least 3 experiments per concentration are performed. The values of each collection period of 1 min are averaged and plotted versus time. The effects of the test compound (increase or decrease of the secreted hormones) are compared with the control periods and the effect of elevated glucose.

CRITICAL ASSESSMENT OF THE METHOD

The isolated perfused rat pancreas has been proven to be a useful tool to study the degree and time course of secretion of the pancreatic hormones insulin, glucagon and somatostatin under the influence of sulfonylureas.

MODIFICATIONS OF THE METHOD

Penhos et al. (1969) described a technique for *in situ* perfusion of **rat** pancreas alone or with a small intestine preparation which is suitable for studying insulin and/or glucagon secretion and the role intestinal factors have upon it.

Silvestre et al. (1996) studied in the perfused pancreas of the rat the effect of $^{8-32}$salmon calcitonin, an amylin antagonist, on insulin, glucagon and somatostatin release.

Weir et al. (1979) used the isolated perfused pancreas of **dogs** to study the influence of glucagon, insulin and arginine on somatostatin and pancreatic polypeptide secretion.

Retrograde perfusion of the isolated perfused pancreas of dogs was used as a model for testing the relative effects of glucose versus insulin on the A cell by Stagner and Samols (1986).

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K.6.1.2 Perifusion of isolated rat pancreatic islets

PURPOSE AND RATIONALE

An assay with isolated pancreatic islets to study the dynamic response and transitions between various metabolic states has been recommended by Idahl (1972). A further description and discussion of the method were given by Malaisse-Lagae and Malaisse (1984).

PROCEDURE

Male Wistar rats weighing 200–250 g serve as donors. Before the study, the animals have free access to food and water. Two donor rats are used for each test. The pancreas is removed under pentobarbital anesthesia. The islets are obtained by the collagenase (e.g. collagenase type IV, Worthington, Freehold) method and collected under a stereomicroscope. In each test, up to 10 chambers each with 15 islets are perifused. Cut-off Microfuge® tubes, sealed with Tuohy-Borst® adapters, serve as perfusion chambers. Two thick-walled, small-diameter Teflon® catheters are passed through the adapter into the chamber. One of the catheters extends to the bottom of the chamber and acts as the perifusate inlet, the other extends to the lower edge of the adapter cone and acts as outlet. The latter is connected to a multi-channel peristaltic pump which delivers the perifusate to a fraction collector. The chamber volume is 0.15 ml. The perifusate flow rate is 0.1 ml/min. The perifusate consists of a carbogenated Krebs-Ringer bicarbonate buffer with 1.0 mmol/l glucose, 0.25% bovine albumin, and 5 mmol/l theophylline. The storage vessels for the perifusate, the chambers, and the inlet catheters are immersed in a water bath of 37 °C.

After a pre-perifusion phase of one hour, the perifusate is collected every min for 46 min. From the 2nd until the 18th min, the test compound is added at concentrations between 0.1 and 2.5 µmol/l, and from the 34th to the 46th min, the glucose concentration is raised to 20.0 mmol/l.

Insulin is determined with the RIAgnost® kit using rat insulin as standard in every second sample. The determination is done immediately after the end of an experiment.

EVALUATION

The raw data are expressed in µU insulin per islet/min. Mean and standard error of the mean of each time interval are calculated for graphical representation. The values under exposure to drug are compared with the values under perifusion with glucose only, and with the effect of elevated glucose.

MODIFICATIONS OF THE METHOD

Lernmark (1974) described a method using free cell suspensions from **mouse** pancreatic islets. The glucose response was found to be lower compared to that of intact isolated islets.

A microperifusion system with especially designed microperifusion chambers was described by Panten et al. (1977).

Details of islet cell purification have been reported by Pipeleers (1984).

Tissue culture of dispersed islet cells has been described by Fletcher and Weir (1984).

A simple method for **human pancreatic β cell cultures** has been described by Yoon et al. (1984).

Islet cell membrane isolation and characterization have been described in detail by McDaniel et al. (1984).

Kaiser and Cerasi (1991) described the long term monolayer culture of adult rat islet of Langerhans as an experimental model for studying chronic modulation of b-cell function.

Horaguchi and Merrell (1981) described a method for preparation of viable islet cells from **dogs**.

This method has been used by Marincola et al. (1983) to study the effect of insulin on its own secretion.

Marchetti et al. (1989) used isolated islets of Langerhans of adult **pig** pancreata obtained from slaughterhouses to evaluate the insulin secretion in response to glucose and arginine and inhibition of insulin secretion by the presence of purified insulin.

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K.6.1.3 Insulin producing tumor cells

PURPOSE AND RATIONALE

Chick et al. (1977) described a transplantable insulinoma in the rat which was originally observed as primary tumor in the pancreas of an old male NEDH albino rat being previously irradiated during a parabiosis experiment. Fragments of this tumor were transplanted to young NEDH rats in many passages inducing severe hypoglycemia in host rats. Gazdar et al. (1980) reported the establishment of a continuous cell line of a rat islet cell tumor which secretes primarily insulin and some somatostatin. Bhatena et al. (1982) studied insulin, glucagon, and somatostatin receptors on cultured cells and clones from rat islet cell tumor.

PROCEDURE

The insulinoma is transplanted when the tumor has reached a diameter of about 2 cm and the carrier animal exhibits distinct manifestations of insulin excess. The tumor is removed, cut open, mixed briefly in a mortar and one tenth to one twentieth is injected between the shoulder blades of another animal. No metastases are observed. The histological examination also shows a nonmalignant adenoma. The tumors grow only in rats of the strain NEDH (New England Deaconess Hospital).

Insulin producing cells from the RINm5F cell line are grown in RPMI medium containing 10% (v/v) heat inactivated fetal calf serum, 50 IU/ml penicillin, 0.25 µg/ml amphotericin B, and 50 µg/ml streptomycin at 37 °C in an atmosphere of humidified air/CO_2 (19 : 1) (Praz et al. 1983). The cells are seeded at a density of 3.5×10^4 cells/ml in 20 ml of medium (75 cm^2 culture flasks). The medium is replaced 4 times a week (one passage). Thereafter, the cells are treated with trypsin (0.02% trypsin in 0.9% NaCl/0.2 mM EDTA) for 2–5 min at 37 °C. The trypsin-treated cells are diluted, re-seeded at a density of 2×10^6 cells per 75 cm^2 culture flask and grown to 70% confluency.

The cells are used for preparation of cell membranes (Müller et al. 1994).

MODIFICATIONS OF THE METHOD

The cell lines **RIN-m** and **RINm5F** have been used by several authors e.g. for receptor studies or for patch clamp experiments (Gögelein et al. 1998). RINm5F

cells were maintained in RPMI 1 640 tissue culture media, containing 11 mM glucose, supplemented with 10% fetal calf serum, 2 mM glutamine and 50 µg/ml gentamycine. Cells were seeded out every 2–3 days onto Petri dishes and kept in a humidified atmosphere of 95% O_2 and 5% CO_2 at a temperature of 37 °C. For patch-clamp experiments, cells were isolated by incubation in a Ca^{2+}-free medium containing 0.25% trypsin for about 3 min. Single cells and clusters of 2–3 cells were obtained after centrifugation with 800 rpm and were stored on ice until use. The tight-seal whole-cell patch-clamp technique was applied to single cells.

Several other insulin secreting cell lines were reported in the literature (Review by Poitout et al. 1996).

The insulin secreting **HIT cell line** was developed by Santerre et al. (1981) by isolating pancreatic islets from the hamster, dispersing the islets into single cells, transforming the cell isolates with the simian virus 40 (SV40), and cloning out the insulin-secreting cell lines. These clonal cells retain a differentiated function and respond to secretagogues and inhibitors of insulin secretion (Boyd et al. 1991).

Experiments for assessment of glucose transport activity in HIT cells and Western blot analysis for GLUT2 in these cells after incubation with glibenclamide and troglitazone were performed by Masuda et al. (1995).

Asfari et al. (1992) derived **INS-1 cells** and **INS-2 cells** from parental RINm5f cells. In the course of a coculture of lymphocytes and RIN cells in the presence of 2-mercaptoethanol, the authors observed the formation of free-floating cell aggregates which appeared to be morphologically different from the parental cells. These clusters were isolated and gave rise to the INS-cell lines, whose viability is dependent on the presence of 2-mercaptoethanol in the media and whose secretory characteristics are similar to those of native islets.

Hanahan (1985) applied gene transfer technology to the establishment of β-cell lines. In order to target the expression of viral DNA to the β-cell, recombinant oncogenes were designed by fusion of the 5' regulatory region of the rat insulin II gene with the early coding region of SV40. The transgenes were microinjected into fertilized mice embryos, which were then implanted in the oviduct of pseudo-pregnant females. The offspring specifically expressed SV40 in their β-cells and spontaneously developed β-cell tumors at 10–20 weeks of age. Tumors were subsequently excised, isolated and propagated in culture, giving rise to β-tumor cell (**β-TC**) lines (Erfrat et al. 1988). Because these cells do not grow in culture at low density, the have not been cloned, and several subpopulations have been derived.

Similarly, the **MIN6 cell** was derived from transgenic mice expressing a comparable transgene (Miyazaki et al. 1990).

Hamaguchi et al. (1991) applied this technique to the development of the **NIT1 cell line** from transgenic NOD mice in order to establish an immortalized source of NOD β-cells.

The **betacyte**, also called the **HEP G2ins/g cell**, is a genetically engineered insulin-secreting human liver cell line that is glucose responsive (Simpson et al. 1995, 1996; Tuch et al. 1997). The clone was constructed by doubly transfecting the HEP G2 cell line with insulin cDNA under the control of the constitutive CMV promoter and the cDNA for the glucose transporter 2, which is specific to β-cells and hepatocytes. This cell is capable not only of synthesizing, storing and secreting insulin in a regulated fashion when challenged with physiological concentrations of glucose, but also of acting as a liver cell and secreting albumin.

Tuch et al. (1997) studied the effect of β-cell toxins (streptozotocin, alloxan and pentamidine) on betacytes.

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K.6.1.4 Isolation of membranes

PURPOSE AND RATIONALE

For receptor binding studies, membranes are isolated from adenoma tissue suspension, cerebral cortex of Wistar rats (Kaubisch et al. 1982; Geisen et al. 1985) or from RINm5F cells (Müller et al. 1994).

PROCEDURE

Cerebral cortex homogenates are prepared from decapitated Wistar rats. The cortices are immersed in ice-cold buffer, pH 7.5, and are homogenized under cooling in an Ultraturrax® tissue homogenizer. The cell membranes are centrifuged at 50 000 *g*, resuspended in fresh phosphate buffer and recentrifuged at the same speed. The resulting pellet is suspended in phosphate buffer, the final dilution being 1 : 50, based on wet weight of the cerebral cortex. One ml aliquot of this cell suspension is used for the incubations.

β-Cell adenoma tissue, dissected from rats of the strain NEDH, is homogenized in 25 mM HEPES/KOH, pH 7.4, 0.25 M sucrose, 0.5 mM EDTA, 100 μM PMSF (50 ml/g tissue) under cooling with ice (3 × 5 s) with an Ultraturrax® homogenizer. After centrifugation (5 min at 3 000 *g*) the supernatant is transferred into new tubes and centrifuged (60 min at 200 000 *g* at 4 °C). The pellet is washed with 2 × 5 ml of the same buffer, resuspended in 2 volumes of the same buffer and centrifuged again (30 min at 75 000 *g*). The pellet is resuspended again in the membrane buffer.

Insulin producing cells from RINM5F cell culture (one culture flask) are washed twice with 25 mM HEPES/KOH (pH 7.4), 0.25 M sucrose, 0.5 mM EDTA, scraped with 20 ml of the same buffer and homogenized with 10 strokes of a tight fitting Potter-Elvehjem homogenizer followed by sonication (bath sonicator, 4 °C, 10 s, maximal power). After centrifugation (45 min at 200 000 *g*, 4 °C) the pellet is suspended in 10 ml of 25 mM HEPES/KOH (pH 7.4), 0.25 M sucrose, 100 mM NaCl, 0.5 mM EDTA, 200 μM PMSF and recentrifuged (10 min at 1 000 *g*). The supernatant is transferred to a new tube and centrifuged (30 min at 50 000 *g*, 4 °C). The pellet is washed once with 25 mM HEPES/KOH (pH 7.4) and finally suspended in membrane buffer (25 mM HEPES/KOH pH 7.4, 150 mM NaCl, 1 mM EDTA, 100 μM PMSF, 10 μg/ml soybean/trypsin inhibitor, 10 μM leupeptin, 1 mM iodoacetamide at 5 mg protein/ml). Aliquots are stored at –80 °C.

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K.6.1.5 Receptor binding of sulfonylureas[4]

PURPOSE AND RATIONALE

Sulfonylureas block ATP-dependent K^+ channels in the β-cell plasma membrane (Schmid-Antomarchi et al. 1987). Binding to the receptor and depolarization of

[4] Contributions by G. Müller.

the membrane initiates a chain of events leading to the release of insulin (Boyd 1992). The high affinity sulfonylurea receptor is considered to be an integral part of the ATP-sensitive K^+ channel (Aguilar-Bryan et al. 1992). Binding studies of sulfonylureas and other drugs can be performed on isolated pancreatic islets, isolated insulinoma cells, isolated intact membranes or solubilized membranes (Geisen et al. 1985; Gaines et al. 1988; Panten et al. 1989, 1992, 1993; Müller et al. 1994).

PROCEDURE

Binding to intact RINm5F cells

Cells seeded in culture flasks at a density of 2×10^6 cells and grown to 70% confluency are treated with trypsin (0.02% trypsin in 0.9% NaCl/0.2 mM EDTA), washed with 3×10 ml HEPES based Krebs-Ringer buffer containing 20 mM HEPES/KOH (pH 7.4), 125 mM NaCl, 5 mM KCl, 7.5 mM $NaHCO_3$, 2 mM $CaCl_2$, 0.8 mM $MgSO_4$, suspended at a density of 2×10^7 cells/ml in the same buffer and then incubated for 45 min at 4 °C at a density of 4×10^6 cells per 0.5 ml assay volume with labelled sulfonylurea, e.g., [^{3}H]glibenclamide or [^{3}H] glimepiride at concentrations between 0.1 and 20 nM. For determination of non-specific binding, 1 µM unlabeled ligand is included. The incubation mixture is rapidly filtered on Whatman GF/C filters soaked with ice-cold buffer under reduced pressure. Filtration and washing has to take less than 30 s. The filters are washed three times with 6 ml ice-cold buffer containing 100 µM PMSF, 0.5 µg/ml leupeptin, 0.75 mg/ml pepstatin, 1 µg/ml aprotinin, 50 mg/ml antipapain dihydrochloride, placed in 10 ml of ACSII scintillation cocktail and after incubation overnight counted for radioactivity in a liquid scintillation counter.

Binding to intact membranes

Filter binding assays are performed in a total volume of 1 ml containing 5 mg membrane protein, 25 mM MOPS/KOH (pH 7.4), 0.1 mM $CaCl_2$ and labelled sulfonylurea, e.g. [^{3}H] glibenclamide or [^{3}H] glimepiride at concentrations between 0.1 and 20 nM. After incubation for 45 min at 25 °C, the binding reaction is terminated by rapid filtration through Whatman GF/F filters soaked with the same buffer. The filters are washed three times with 5 ml of ice-cold 25 mM HEPES/KOH (pH 7.4), 100 mM NaCl, 1 mM EDTA, 200 µM PMSF, 0.5 µg/ml leupeptin, 0.75 µg/ml pepstatin and two times with 5 ml of ice-cold HEPES/KOH (pH 7.4), placed in 10 ml of ACSII scintillation cocktail and after incubation overnight counted for radioactivity in a liquid scintillation counter. Non-specific binding is determined in parallel samples in the presence of 1 µM unlabeled ligand.

Binding to solubilized membranes

Isolated β-cell tumor membranes are diluted with 25 mM MOPS/KOH (pH 7.4), 100 mM NaCl, 0.1 mM $CaCl_2$, 200 µM PMSF, 0.5 µg/ml leupeptin, 0.75 µg/ml pepstatin, 50 µg/ml antipapain dihydro-chloride, 1 mg/ml aprotinin at 1 µg protein/ml and solubilized by addition of 1/10 vol. of 10% digitonin and subsequent incubation for 30 min at 4 °C. After centrifugation for 30 min at 200 000 *g* and 4 °C, the supernatant is incubated with the radioactive ligand, e.g. [^{3}H] glibenclamide or [^{3}H] glimepiride at concentrations between 0.1 and 20 nM in a final volume of 1 ml. After 30 min at 25 °C, 3 ml of ice-cold PEG 8 000, 25 mM MOPS/KOH (pH 7.4), 100 mM NaCl, 0.5 mM EDTA, 200 µM PMSF is added. After further incubation for 15 min at 4 °C, the incubation mixture is filtered through nitrocellulose filters (Millipore, HAWP, 0.22 µm), soaked with 5 ml ice-cold 10% PEG 8 000 and 4 times 5 ml 6% PEG 8 000, dried, placed in 10 ml of ACSII scintillation cocktail and counted for radioactivity in a liquid scintillation counter. From all values, radioactivity retained on the filter in the absence of protein during the binding reaction is subtracted (up to 1% of total radioactivity added). Non-specific binding is determined in the presence of 1 µM unlabeled ligand.

Binding to intact HIT-T15 cells

Cultured cells producing the β-cell sulfonylurea receptor, SUR1, include the hamster insulin secreting tumor cell line, HIT-T15 (passage 65–75; CRL1 777 ATCC, Rockville, MD). Cells are maintained in T-175 culture flasks (Falcon) as monolayers in DMEM containing high glucose supplemented with 10% fetal bovine serum, 100 U, 7 ml penicillin and 0.1 mg/ml streptomycin. Cells are grown in 5% CO_2 at 37 °C, maintained in subconfluent cultures, fed three times a week, and subcultured as needed. To subculture, confluent cells are detached with 0.05% trypsin/EDTA, resuspended in supplemented DMEM/-high glucose and replated at 1/10 of the original density.

Kinetic studies

For studying the association kinetics, the binding reaction is started by addition of radiolabeled ligand and terminated after predetermined periods of time by rapid filtration. For studying the dissociation kinetics, displacement of radiolabeled ligand at equilibrium (60 min incubation) is initiated by addition of unlabeled drug (final concentration 1 µM) and terminated after given periods of time. For termination, the incubation mixtures are rapidly chilled to 2–4 °C by placing the assay tube in a dry ice/methanol bath for 1 s immediately prior to filtration in a filtration apparatus located in a cool bench.

EVALUATION

K_d as well as B_{max}-values are calculated from Scatchard plot analyses and K_{on} and K_{off} values from kinetic studies. Curvilinear Scatchard plots allow the assumption of more than one binding site. IC_{50} values for half maximal inhibition of [^{3}H] glibenclamide binding can be calculated from competition-inhibition plots.

MODIFICATIONS OF THE METHOD

Masuda et al. (1995) tested the effect of troglitazone on sulfonylurea receptor binding in rat pancreatic islet cells and in HIT cells (a hamster β-cell line). They concluded that troglitazone has a non-competitive binding site at, or in the vicinity of, the sulfonylurea receptor.

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K.6.1.6 Interaction of sulfonylureas with β-cells

PURPOSE AND RATIONALE

It is now established that increasing the closed probability of the β-cell ATP-dependent potassium channel (K_{ATP}) is the major mechanism through which sulfonylureas as well as glucose (via transport, phosphorylation and metabolism for generation of ATP) stimulate insulin release from pancreatic β-cells (Ashcroft and Ashcroft 1992). The resulting reduction in potassium ion efflux causes depolarization of the β-cell plasma membrane which in turn leads to opening of voltage-sensitive Ca^{2+} channels of the L-type. The increased influx of Ca^{2+} and thus the elevated cytosolic Ca^{2+} levels trigger the fusion of insulin-containing secretory granules with the plasma membrane, presumably mediated by Ca^{2+}/calmodulin-dependent protein kinase and additional unknown mechanisms (Philipson 1995; Aquilar-Bryan and Bryan 1999). The β-cell K_{ATP} consists of the regulatory sulfonylurea receptor subunits (SUR), such as SUR1, and the physically associated pore-forming potassium ion inwardly rectifying subunit, KIR6.2, in a multimeric assembly with the SUR and KIR6.2 subunits in 1 : 1-stoichiometry and four identical SUR/KIR6.2 complexes per functional channel unit (size of the channel holocomplex $[SUR/KIR6.2]_4$ about 1 000 kDa; Skeer et al. 1994; Clement et al. 1997; Bryan and Aquilar-Bryan 1999). SUR1, which is predominantly expressed in neuronal/pancreatic β-cells, is an ATP-binding cassette (ABC) protein or transport ATPase, which closely resembles members of the multidrug resistance associated protein family with 17 predicted transmembrane domains (TMD) and two nucleotide-binding folds (NBF), which bind specifically (Mg^{2+})ATP/ADP (Bryan et al. 1995; Inagaki et al. 1996; Ueda et al. 1999). The KIR6.2 subunits have two TMDs which somehow contribute to the K^+ conductivity and selectivity (Babenko et al. 1998). The glibenclamide-binding site of SUR1 is proposed to consist of a benzamido (meglitinide)-binding site on TMDs

1–5 and the sulfonylurea (tolbutamide)-binding site on TMDs 12–17 based on photolabeling with [^{125}I]iodoglibenclamide and chimeric receptors (Ashfield et al. 1999; Babenko et al. 1999). Interaction with both the benzamido- and sulfonylurea-binding sites could account for the several thousand-fold increase in affinity of glibenclamide vs. tolbutamide. SUR2A/B are splice variants of a single SUR2 gene differing in 42 amino acids at the carboxy-terminus, exclusively, with SUR2A expressed mainly in cardiac/skeletal muscle cells and SUR2B in vascular/non-vascular smooth muscle cells (Aquilar-Bryan et al. 1998). K^+-channel openers (KCOs) bind to SURs at the tolbutamide-binding site encompassing those (flanking) TMDs 12–17 which surround the central core region of the tolbutamide-binding site (Uhde et al. 1999). SURs define the sensitivity of the K_{ATP} channel holocomplex for its sensitivity toward both sulfonylureas and KCOs with SUR1 mediating high sensitivity for sulfonylureas/low sensitivity toward KCOs and vice versa SUR2A/2B medi-ating low sensitivity for sulfonylureas/high sensitivity for KCOs. Chimeric SURs can be engineered by recombinant DNA technology (harboring TMDs 12–17 from both SUR1 and SUR2A/B) with high sensitivity for both sulfonylureas and KCO arguing for a modular structural and functional organization of SURs (Uhde et al. 1999).

The cooperative interactions between NBFs 1 and 2 of SUR1 in the open state of the K_{ATP} channel are disrupted by the conformational change induced by glibenclamide-binding which encompasses TMD 12–17 and especially TMD 1–5 (Babenko et al. 1999). This conformational change repositions TMD 2 of KIR6.2 (M2), which is in close contact to TMD 1–5 of SUR1, to a closed state via a mechanism that requires the intact N-terminus of KIR6.2. Thus sulfonylurea-binding to SUR1 apparently induces a subtle relaxation of the intimate association between SUR1 and KIR6.2, which is required for keeping KIR6.2 in a (partly) open state. Compatible with this simplified view are recent findings that certain mutations in both SUR1 and KIR6.2 cause recessive forms of familial hyperinsulinism or persistent hyperinsulinemic hypoglycemia of infancy due to permanent closure of K_{ATP} presumably in course of weakening of the interaction between SUR1 and KIR6.2 to a certain degree (Thomas et al. 1995; Thomas et al. 1996; Kane et al. 1996). Binding of ATP to the carboxy-terminal domain of KIR6.2 facilitates and/or stabilizes the glibenclamide-induced relative dissociation of SUR1 and KIR6.2 and thereby favors the closed state of the K_{ATP} which may explain (in part) the stimulation of glucose- (i.e. ATP-) induced insulin release by sulfonylureas. In contrast, interaction of ATP with NBF-1 and Mg^{2+}ADP with NBF-2 is relieved upon glibenclamide binding.

PROCEDURE

Analysis of recombinant SUR1

Heterologous expression of SUR1

Chinese hamster ovary and COS (1, m6, 7) cell lines do not produce SUR1 and are used for heterologous SUR1 expression. For transient transfection, COS cells are plated at 50–60% confluence. Cells are transfected using either a DEAE-dextran or a lipofectamine protocol. 3-day-old cultures of COS cells are trypsinized and replated at a density of 3.5×10^5 cells per 35-mm well (six-well dish) and allowed to attach overnight. Typically, 5 µg of a SUR1 plasmid is mixed with 5 µg of a KIR6.2 plasmid and brought up to 7.5 µl final volume in TBS (8 g/l NaCl, 0.4 g/l KCl, 0.2 g/l Na_2HPO_4, 3 g/l Tris base, 0.2 g/l $CaCl_2$, 0.1 g/l $MgCl_2$, pH 7.5) before addition of DEAE-dextran (30 µl of a 5 mg/ml solution in TBS). The samples are vortexed, collected by spinning in a microfuge, then incubated (15 min, 22 °C) prior to addition of 0.5 ml of 10% FBS in TBS. Cells are washed twice with Hanks' balanced salt solution (HBSS), the DNA mix is added, and the cells are maintained in a 37 °C CO_2 incubator. After 4 h incubation, the DNA mix is decanted and the cells shocked for 5 min in 1 ml HBSS + 10% dimethyl sulfoxide, then placed in 2 ml of DMEM/high glucose containing 2% FBS and 10 µM chloroquine and kept in the incubator for 4 h. Thereafter the cells are washed three times with HBSS and incubated in normal growth media until assayed.

Alternatively, lipofectamine is used for transfection instead of DEAE-dextran. COS cells are plated in six-well dishes (see above) and are used at 70–80% confluency. Typically 1 µg of a SUR1 plasmid and 1 µg of a KIR6.2 plasmid are mixed with 375 µl of Opti-Mem reduced serum medium (Life Technologies, Inc.), then added to 375 µl of Opti-Mem containing 9 µl of lipofectamine. After 1 h incubation, the mixture is supplemented with Opti-Mem to 1 ml final volume and added to the cells, which had been washed twice with 3 ml of Opti-Mem each. After 5 h incubation, the Opti-Mem is replaced with DMEM/high glucose containing 10% FBS. Transfections are scaled up based on the area of the plates used. For instance, 150-mm plates used for membrane isolations are transfected with 100 µg of each plasmid using the DEAE-dextran protocol. Transfected cells are used for determination of sulfonylurea binding and photolabeling or $^{86}Rb^+$ efflux 36–72 h posttransfection.

Membrane isolation from HIT-T15 cells

Membranes are prepared from 15–25 150-mm dishes. Cells are washed three times in phosphate-buffered saline (PBS), pH 7.4, then scraped in PBS and collected

in 10-ml plastic tubes. The cells are pelleted, resuspended in 10 ml of hypotonic buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA), and allowed to swell for 1 h on ice. Cells are then homogenized, transferred to a 15-ml glass tube, and spun at 2 000 *g* for 20 min at 4 °C to remove nuclei and unbroken cells. The supernatant is then transferred to a polycarbonate centrifuge tube and membranes are collected by centrifugation (50 000 rpm, 90 min, Ti80 fixed-angle rotor). The pelleted membranes are resuspended in 5–200 µl of membrane buffer (25 mM Tris/HCl, pH 7.4, 2 mM EDTA, 250 mM sucrose, 0.2 mM PMSF, 10 µg/ml leupeptin) and stored frozen in aliquots in liquid N_2.

Membrane solubilization

Membranes are rapidly thawed, resuspended using a Teflon-in-glass homogenizer, and mixed with ice-cold digitonin (25% w/v, in deionized water, prepared daily) to a final protein concentration of 3 mg/ml and 1% digitonin. Subsequent steps are performed at 25 °C in the presence of 0.2 mM PMSF, 10 µg/ml leupeptin, 20 µg/ml pepstatin, 0.2 mM phenanthroline). After 30 min of incubation, solubilized membrane proteins are separated from insoluble material by centrifugation (100 000 *g*, 1 h, 4 °C). Alternatively, the thawed and rehomogenized membranes are mixed with the same volume (resulting in 3 mg/ml protein) of ice-cold 0.2% (w/v) phosphatidylcholine, 20% (w/v) glycerol, 280 mM KCl, 4 mM EDTA, 0.4 mM PMSF, 20 µg/ml leupeptin, 50 mM Hepes/KOH, pH 7.4, 2.5% (w/v) Triton X-100 or CHAPS, incubated on ice for 1 h (under gentle stirring) and then centrifuged (150 000 *g*, 1 h, 4 °C). The supernatants are collected and stored frozen in liquid N_2.

Partial purification of SUR1

For purification of the 140-kDa core glycosylated species of SUR1, 4-ml aliquots of digitonin-solubilized membranes are cycled for times over a 1-ml concanavalin A-Sepharose column equilibrated with 40 mM Tris/HCl (pH 7.5), 0.2 M NaCl, 1 mM EDTA, 1% digitonin. The column is washed with 8 ml of the equilibrating buffer and eluted with 4 ml of the equilibrating buffer containing 0.5 M methyl α-methylmannopyranoside. The eluted protein is stored in liquid N_2. For purification of the 150-kDa complex glycosylated SUR1, wheat germ agglutinin-Sepharose is used instead of concanavalin A-Sepharose. The procedure is the same as described with the exception of elution of the receptor using 0.3 M N-acetylgluosamine.

The eluate for the lectin columns is cycled twice over a 1-ml column of Reactive Green 19-agarose equilibrated with 40 mM Hepes/KOH (pH 8.5), 1 mM EDTA, 0.5% (w/v) digitonin. After washing with 10 ml of equilibrating buffer, and 10 ml of the same buffer supplemented with 0.5 M NaCl, the protein is eluted with 4 ml of equilibrating buffer containing 1.5 M NaCl. The eluate from the Reactive Green-19 purification step is diluted 1 : 2 with 40 mM Hepes/KOH (pH 8.5), 1 mM EDTA, 0.2% digitonin, then cycled twice over a 1-ml phenylboronate-10 Sepharose column. The phenylboronate column is washed with 10 ml of the HEPES buffer, followed by 2 ml of 0.1 M Tris/HCl (pH 7.5), 1 mM EDTA, and 0.2% digitonin. The protein is eluted with 4 ml of 0.1 M Tris/HCl (pH 7.5), 1 mM EDTA, 0.15% (w/v) sodium dodecyl sulfate. Prior to binding measurements, pooled samples from the various column steps are concentrated by centrifugation (3 000 *g*, 30 min, 4 °C) through 100 000 molecular weight cut-off filters (Amicon), which have been pretreated with 5% (v/v) Tween 20 for 14 h at 4 °C.

Analysis of SUR1 in extrapancreatic tissues

In addition to β-cells and hypothalamic neurons, SUR1 is also expressed in human adipocytes (Shi et al. 1999). Closure of the K_{ATP} by sulfonylurea binding to the adipocyte SUR1 leads to depolarization of the plasma membrane, opening of voltage-dependent Ca^{2+} channels and concomitant increase of the cytosolic Ca^{2+} concentration. Thus, adipocytes and determination of intracellular Ca^{2+} may be used for assaying the potency of sulfonylureas with regard to binding to SUR1.

Prior to fluorometric Ca^{2+} measurement, the adipocytes are washed three times (by flotation) with HBSS (140 mM NaCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 4 mM $NaHCO_3$, 5 mM glucose, 2 mM sodium pyruvate, 2 mM glutamine, 20 mM Hepes, 1% BSA). The cells are then loaded with Fura-2-acetoxymethyl ester (10 µM) in the same buffer for 45 min at 37 °C in the dark with continuous shaking. For removal of extracellular dye, the cells are washed three times with HBSS and resuspended in HBSS at 3.5×10^5 cells/ml. Cytosolic Ca^{2+} is determined using dual excitation (340 and 380 nm) and single emission (510 nm) fluorometry. After establishment of a stable baseline in the absence or presence of the K_{ATP} channel openers, diazoxide and nitrendipine, the response to sulfonylureas is determined. Digitonin (25 µM) and Tris/EGTA (100 mM) are used to measure maximal and minimal fluorescence to calibrate the signals. Cyto-solic Ca^{2+} is calculated by the equation of Grynkiewicz et al. (1985).

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K.6.1.7 Photoaffinity labeling of β-cell membranes[5]

PURPOSE AND RATIONALE

Binding sites of sulfonylureas have been identified in pancreatic β-cell membranes and in other tissues such as brain (Bernardi et al. 1988). The technique of photoaffinity labeling allows the identification and purification of membrane receptors (Yip 1984; Kramer et al. 1994).

PROCEDURE

For photoaffinity labeling of β-cell membranes, samples of membrane suspension are prepared as described above. 600 µg of β-cell membranes, suspended in 100 mM sodium phosphate buffer (pH 7.4), are incubated in a total volume of 200 µl with 40–60 nM (0.3–0.4 µCi) [^{3}H]glibenclamide at 20 °C in the dark for 60 min. Irradiation is performed in a Rayonet RPR 100 photochemical reactor (Southern Ultraviolet Co., Hamden, CT), equipped with RPR 2 530 Å lamps at a distance of 10 cm from the lamps. After irradiation at 254 nm for 2 min the membranes are diluted with 1 ml of 10 mM Tris-HEPES buffer (pH 7.4) containing 4 mM EDTA, 4 mM iodoacetamide, 4 mM PMSF and centrifuged at 48 000 *g* for 30 min. The resulting pellet is resuspended in 200 µl water and protein precipitated according to Wessel and Flügge (1984).

For inhibition of [3H]glibenclamide photo-labeling of the receptor protein by test compounds as compared with glibenclamide, 600 µg of β-cell membranes are incubated with various concentrations (10^{-9} to 10^{-4} M) of glibenclamide or the test compounds. After incubation with 60 nM (0.37 µCi) [^{3}H]glibenclamide in the dark, the membranes are photolabeled at 254 nm for 2 min. After washing of the membranes, the proteins are separated by SDS gel electrophoresis.

For SDS gel electrophoresis, the protein precipitates are dissolved in 70 µl of 62.5 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 5% mercaptoethanol, 0.005% bromophenol blue by shaking on a mixer for 60 min. After centrifugation at 15 000 g for 10 min the clear supernatants are subjected to SDS gel electrophoresis on 150 × 180 × 1.5 mm slab gels (Kramer et al. 1983). After fixing and staining, the gels are scanned with a CD-50 densitometer (Desaga, Heidelberg). The

[5] Contributions by G. Müller.

radioactivity is determined by liquid scintillation counting after slicing the gels into 2 mm pieces and after digestion of proteins with Biolute.

EVALUATION

The radioactive peak of [^{3}H]glibenclamide bound to the receptor protein is decreased depending on the concentration of unlabeled glibenclamide or test compounds. IC_{50} values can be calculated.

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K.6.1.8 $^{86}Rb^+$ efflux measurements

PURPOSE AND RATIONALE

The binding of sulfonylureas to the receptor inhibits ATP-sensitive K^+ channels, decreasing the K^+ efflux and leading to membrane depolarization. This opens voltage-gated calcium channels. The rise in [Ca^{2+}] triggers the exocytosis of insulin. $^{86}Rb^+$ efflux can be used as marker for K^+ efflux.

PROCEDURE

RINm5F cells are grown and plated at a density of 200 000 cells/well (Falcon 24-well tissue culture plates). $^{86}Rb^+$ efflux experiments are performed at 37 °C and overnight equilibration of cells in RPMI 1 640 medium supplemented with 10% fetal calf serum, 0.1 µCi/ml ^{86}RbCl, and 0.2 µCi/ml L-[^{3}H]leucine (internal marker of cell recovery). After removing the medium, cells are preincubated in a medium containing 120 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM KCl, with 20 mM HEPES/NaOH buffer, at pH 7.5, supplemented with 0.1 µCi ^{86}RbCl, 0.24 mg/ml oligomycin, 1 mM 2-deoxy-D-glucose and several concentrations of various sulfonylureas. $^{86}Rb^+$ efflux studies are initiated by removing the pre-incubation medium and incubating the cells with 200 µl of the same medium/well without $^{86}Rb^+$, oligomycin, and 2-deoxy-D-glucose. Efflux is stopped by removing this medium and washing the cells three times with 1 ml of 0.1 M $MgCl_2$ at 37 °C. Cells are extracted with 2 × 1 ml of 0.1 N NaOH and counted. Total intracellular concentrations of ATP are measured after extracting the cells with 1% Triton X-100 by using the luciferase-luciferin technique.

EVALUATION

Inhibition is measured as percent of maximum $^{86}Rb^+$ efflux. Half maximum inhibition constants can be calculated.

MODIFICATIONS OF THE METHOD

Daniel et al. (1991) recommended a high through-put ^{86}Rb efflux assay in human medulloblastoma cells TE671 for screening of potassium channel modulators.

Hu et al. (1995) cloned a voltage-regulated K channel from human hippocampus for transfection into Chinese hamster ovary (CHO) cells. The authors recommended this method as a high through-put assay to identify isotype-specific K-channel modulators.

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K.6.1.9 Membrane potential (measurement of the ATP sensitive potassium channel by voltage-clamp technique)

PURPOSE AND RATIONALE

The insulin secretion of β-cells is regulated by ATP sensitive K^+ channels (Bryan and Aguilar-Bryan 1997) which act as a switch that responds to glucose or sulfonylureas to initiate depolarization (Boyd 1992). This depolarization opens voltage-dependent calcium channels to increase the amplitude of free cytosolic Ca^{2+} levels, which triggers exocytosis of insulin (Nelson et al. 1987). The depolarization can be measured with the voltage-clamp technique.

PROCEDURE

Rat insulinoma cells RINm5F (Gögelein et al. 1998) or cultured pancreatic β-cells from NMRI mice (Rorsman and Trube 1985; Zünkler et al. 1985) or HIT T15 β-cells (Niki et al. 1989) are used. Whole cell patch-clamp experiments are performed according to Hamill et al. (1981), Rajan et al. (1993), Lindau and Neher (1988) at room temperature with cells bathed in an external solution of 140 mM NaCl, 4 mM KCl, 10 mM HEPES, pH 7.4. The patch pipettes are mounted on a suction pipette holder. The outlet is connected to silicon rubber tubing through which the suction is applied. The pipette solution contains 135 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, 0.3 mM NaATP, pH 7.5. Pipettes are prepared by pulling from borosilicate glass, coated with silicon rubber, and heat polished at the tip. Pipette resistances are ranging between 4 and 7 MΩ. A patch-clamp amplifier (e.g. EPC 7, List Electronic, Darmstadt, FRG) is used which allows capacitance and series resistance compensation. The series resistance after achieving the whole cell recording configuration is 5–20 MΩ and 50% series resistance compensation is used to keep the voltage error during current flow below 2 mV. The cell membrane potential is held at –70 to –80 mV and hyper- and depolarizing voltage pulses of 10 mV amplitude are applied alternatively every 2 s. Voltage-dependent currents, i.e., the Ca^{2+} inward current and the delayed K^+ outward current are not activated by these low pulse amplitudes and, therefore, most of the current is flowing through ATP-dependent K^+ channels. Inside-out patches are prepared according to Hamill et al. (1981) by disrupting the outer vesicle membrane.

EVALUATION

The current and voltage signals are stored on magnetic tape or filtered by a 4-pole Bessel filter and displayed on a digital oscilloscope. Outward currents flowing from the cell to the bath are indicated by upward deflections and inward currents by downward deflections. Half maximal effective concentrations are calculated as EC_{50} values.

MODIFICATIONS OF THE METHOD

Henquin and Meissner (1984), Henquin et al. (1984, 1884), Meissner (1990) measured membrane potential of mouse pancreatic β-cells.

Hu et al. (2000) compared the effects of netaglinide, a non-sulfonylurea hypoglycemic agent, with sulfonylureas on pancreatic β-cell K_{ATP} channel activity with the whole-cell configuration of the patch-clamp technique in primary cultures of rat pancreatic β-cells.

Wang and Giebisch (1991) reported a dual modulation of the renal ATP-sensitive K^+ channel by protein kinases A and C in cells of the cortical collecting duct of rabbit kidneys using the cell-attached and inside-out modification.

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K.6.2 Effects on liver

K.6.2.1 Perfusion of isolated rat liver

PURPOSE AND RATIONALE

The technique of liver perfusion has been described extensively by Ross (1972), mentioning in his introductory remarks that the isolated liver was first used by Claude Bernard in 1885. Derived from several modifications, the perfusion of rat liver from the portal vein is the most widely used technique.

PROCEDURE

Male Wistar rats weighing 200–250 g are anesthetized with 150 mg/kg hexobarbital intraperitoneally. After opening the abdomen, two ligatures are tied around the stomach, one around the esophageal end to include the adjacent blood vessels and the other around the pylorus. The stomach is removed between these ligatures, a procedure that facilitates the remaining dissection and the subsequent removal of the liver from the animal. A ligature is placed around the bile duct. The portal vein is tied and cannulated with PP10 tubing. The thorax is opened and the vena cava is cannulated via the right atrium. The lower vena inferior cava is tied and the liver is removed from the animal. From the portal vein the liver is washed with 100 ml hepari-

nized (5 IU/ml) physiological saline solution at 37 °C for 3 min whilst the outflow occurs via the vena cava. The preparation is then transferred to a perfusion apparatus, where the portal vein cannula is attached to tubing containing the oxygenated medium.

Krebs-Ringer bicarbonate buffer with 25% bovine erythrocytes, 1.6% bovine serum albumin, and 22.5 mmol/l Na-L-lactate is used as perfusion medium. The perfusion rate is 30 ml/min. Seventy ml are used for recirculation over 2 h. The test compounds are added in a concentration of 40–100 µmol/l to the perfusate medium.

The central element of the perfusion apparatus is a gas-tight, thermostatized, double-walled suction filter with an insertable sieve base as support of the organ. The discharge tube is elongated with a Plexiglas® tube of 18 cm length and 10 mm inside diameter. The lower end of this tube is connected to a peristaltic pump by means of a Luer® safety joint. The suction filter for perfusion of the liver has an internal diameter of 95 mm. On the return of the perfusate to the organ, it passes through a heat exchanger (glass spiral) and a filter holder with a sieve membrane of stainless steel (diameter 25 mm, mesh size 50 µm). A variable carbogen/oxygen mixture, the ratio of which is depending on the pH value of the perfusate, is used for gassing. The perfusate in the Plexiglas tube is bubbled with 70 ml gas mixture/min. To avoid foam formation, a detergent (14 µl/ml 0.1% Genapol® PF-10) has to be added to the perfusate. Samples for analyses are withdrawn by catheter immediately in front of the Luer joint in the Plexiglas tube.

EVALUATION

Several parameters can be determined in the effluate, such as net glucose production from lactate and net lactate utilization. The values are plotted against time before and after addition of the test compounds, such as insulin or sulfonylureas.

MODIFICATIONS OF THE METHOD

Alexander et al. (1992) studied hepatic blood flow regulation in an isolated dual-perfused rabbit liver preparation.

Alexander et al. (1995) described a miniaturized perfusion circuit using a novel design of organ bath, to maintain a buoyant preparation, and a high-efficiency miniaturized membrane tubing oxygenator for testing hepatic function during prolonged isolated rat liver perfusion.

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K.6.2.2 Isolated hepatocytes

PURPOSE AND RATIONALE

Isolated hepatocytes can be used to study the effect of drugs on hepatic gluconeogenesis and other hepatic metabolic reactions such as ketone body formation and the tricarboxylic acid cycle.

PROCEDURE

Male Wistar rats weighing 200–300 g are used. The hepatocytes are isolated by perfusion of the liver with collagenase (Berry and Friend 1969; Alvarez et al. 1987). The viability of the isolated hepatocytes can be evaluated by the Trypan blue test; usually 90–95% of the cells exclude the stain. The isolated hepatocytes (20×10^6 cells/vial) are suspended in 3.0 ml of Krebs-Ringer bicarbonate buffer containing 4% bovine albumin (pH 7.4). The cell suspension is pre-incubated for 15 min at 37 °C in a Dubnoff metabolic shaking incubator gassed with carbogen.

The following substrates are added in various combinations, and each sample is incubated for 60 min: (1) alanine, fructose, glycerol, lactate, pyruvate (10 mM), or (2) palmitate (0.5 mM, as sodium palmitate bound to albumin). Test drugs, such as sulfonylureas and biguanides are added in concentrations between 0.05 and 5.0 mM.

At the end of the 60-min incubation period, 0.2 ml of 70% $HClO_4$ is added into the medium to stop the reaction. The reaction mixture is then centrifuged, and the supernatant obtained is used to determine the intermediate metabolites. Glucose is assayed by the glucose-oxidase method; lactate (Gutmann and Wahlefeld 1974), pyruvate (Czok and Lamprecht 1974), acetoacetate (Mellanby and Williamson 1974), and β-hydroxybutyrate (Williamson and Melanby 1974) by enzymatic methods.

In order to measure the influence on the tricarboxylic acid cycle, (U-^{14}C) alanine, (1-^{14}C) glutamate,

(1-^{14}C) pyruvate, (1-^{14}C) palmitate, or (1-^{14}C) glucose is added after pre-incubation together with various combinations of compounds and substrates, and incubated for 60 min. The radioactivity is measured by the $^{14}CO_2$ capturing method (Gliemann 1965).

EVALUATION

Values are expressed as means ±SEM. Means are compared by the Student *t*-test.

MODIFICATIONS OF THE METHOD

Agius et al. (1986) isolated parenchymal hepatocytes from male Wistar rats by collagenase perfusion of the liver to prepare hepatocyte monolayer cultures to study regulation of ketogenesis, gluconeogenesis, and glycogen synthesis by insulin and proinsulin.

Fukuda et al. (1992) used primary cultures of rat hepatocytes to study nutritional and hormonal regulation of mRNA levels of lipogenic enzymes, such as acetyl-CoA carboxylase, fatty acid synthase, malic enzyme, and glucose 6-phosphate dehydrogenase.

Instead of isolated hepatocytes, cultures of Hep G2 cells can be used. The Hep G2 line is a minimal deviation of human hepatoma that maintains liver cell morphology and function (Podskalny et al. 1985). Hep G2 cells express insulin receptors, display a number of metabolic responses to insulin and insulin-like growth factor I (Wade et al. 1988; Verspohl et al. 1988) and have been used for various studies (Forsayeth et al. 1986, 1988; Caro et al. 1988). Ciaraldi et al. (1990) found that troglitazone (CS-045) increased glycogen synthase I activity in Hep G2 and BC3H-1 muscle cells.

Benelli et al. (1994) used Zajdela rat ascites hepatoma cells to demonstrate the role of protein kinase C in the activation of the pyruvate dehydrogenase complex by insulin.

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K.6.2.3 Fructose-2,6-bisphosphate production in rat hepatocytes

PURPOSE AND RATIONALE

Glycogenesis in hepatocytes is regulated by the cytosolic level of fructose-2,6-bisphosphate (Van Schaftingen 1993). Insulin induces an increase of fructose-

2,6-bisphosphate through inhibition of a proteinkinase A-dependent phosphorylation of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (Richards and Uyeda (1982), Gabbay and Lardy (1987), Pilkis and El-Maghrabi (1987). Hepatic fructose-2,6-bisphosphate is elevated by various sulfonylureas (Hatao et al. 1985; Kaku et al. 1986; Mori et al. 1992). Moreover, CS-045 (troglitazone), a non-sulfonylurea oral antidiabetic agent, stimulated fructose-2,6-bisphosphate production in rat hepatocytes (Murano et al. 1994).

PROCEDURE

A suspension of hepatic cells is adjusted to 10^6 cells/ml and pre-perfused with Hanks 10 mM-HEPES buffer solution (pH 7.5, containing 0.5% BSA and 1 mM palmityl oleate) at 37 °C for 10 min. The cells are then centrifuged at 40 *g* for 15 s, and the supernatant is removed. To the remainder, the same buffer and various concentrations of the sulfonylurea are added and the mixtures are then incubated at 37 °C for 10 min. The reaction is stopped by cooling on ice. The mixture is centrifuged at 170 *g* at 4 °C for 60 s. The pellet is homogenized with buffer solution containing 1 mM EGTA and 10 mM $MgCl_2$. After heating at 80 °C for 20 min, the homogenate is mixed with an equal volume of 400 mM Tris-HCl buffer (pH 7.5), and the mixture is centrifuged at 17 500 *g* at room temperature for 5 min. In the supernatant fructose-2,6-bisphosphate is measured according to the method of Furuya and Uyeda (1980) by adding a solution of 54 mM Tris-HCl, 1 mM fructose-6-phosphate, 0.2 mM NADH, 7.5 mM dithiothreitol, 0.5 mM EDTA, 0.01 U phosphofructokinase, 0.4 U aldolase, 1 U glycerin-3-phosphate dehydrogenase, and 3 U triosephosphate isomerase. The concentration of fructose-2,6-bisphosphate in the sample solution is measured by colorimetry and compared with the reaction rate of a known concentration of fructose-2,6-bisphosphate.

EVALUATION

Dose-response curves of fructose-2,6-bisphosphate content of isolated hepatocytes after various concentrations of test compound and standard (tolbutamide) are established.

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K.6.3 Effects on muscle

K.6.3.1 Perfused hind limb in rats

PURPOSE AND RATIONALE

Ruderman et al. (1977) proposed the use of the isolated perfused hindquarter of the rat for the study of muscle metabolism.

PROCEDURE

Female Wistar rats weighing 170–230 g are starved 48 h before the experiment. They are anesthetized by i.p. injection of 50 mg/kg pentobarbital. After an midline incision, the skin is reflected and the superficial epigastric vessels are ligated. The abdominal wall is then incised from the pubic symphysis to the xyphoid process using electrocautery. After ligation of the uterine, ovarian and inferior mesenteric arteries, the upper half of the uterus, the ovaries and part of the descending colon are excised, together with adhering adipose tissue. Next, branches of the hypogastric and pudoepigastric trunks supplying pelvic viscera are ligated. Ligatures are also placed around the neck of the bladder and the residual portions of the uterus and descending colon. Adipose tissue in the perineal and retroperitoneal regions is removed.

Two pairs of ligatures are placed around the aorta and the vena cava, one just above the origin of the iliolumbar vessels and the other above the origin of the renal vessels. The inferior epigastric, iliolumbar and renal vessels are then ligated as are the coeliac axis and the portal vein. A ligature is also placed around the tail. The ligatures previously placed around the vena cava and aorta above the origin of the renal vessels are then tied. The aorta is incised between the left renal and iliolumbar vessels and a no. 18 polyethylene catheter filled with 0.85% NaCl containing 200 units of heparin/ml is introduced, passed to a point midway between the iliolumbar vessels and the aortic bifurcation, and after flushing with heparin-NaCl solution finally tied in place. The vena cava is cannulated with a no. 16 needle which is secured in a position so that its tip is at the same level as the aortic catheter. The needle is connected with a transparent vinyl tubing. The preparation is then transferred to a perfusion apparatus, where the aorta cannula is attached to tubing containing the oxygenated medium.

Krebs-Ringer bicarbonate buffer with 25% bovine erythrocytes, 4% bovine serum albumin, and 10 mmol/l D-glucose is used as perfusion medium. The perfusion rate is 8 ml/min. Seventy ml are used for recirculation over 2 h. The test compounds are added in a concentration of 40–100 µmol/l to the perfusate medium.

The central element of the perfusion apparatus is a gas-tight, thermostatized, double-walled suction filter with an insertable sieve base as support of the organ. The discharge tube is elongated with a Plexiglas® tube of 18 cm length and 10 mm inside diameter. The lower end of this tube is connected to a peristaltic pump by means of a Luer® safety joint. The suction filter for perfusion of the hind limb has an internal diameter of 145 mm. On the return of the perfusate to the organ, it passes through a heat exchanger (glass spiral) and a filter holder with a sieve membrane of stainless steel (diameter 25 mm, mesh size 50 µm). A variable carbogen/oxygen mixture, the ratio of which is depending on the pH value of the perfusate, is used for gassing. The perfusate in the Plexiglas tube is bubbled with 70 ml gas mixture/min. To avoid foam formation, a detergent (14 µl/ml 0.1% Genapol® PF-10) has to be added to the perfusate. Samples for analyses are withdrawn by catheter immediately in front of the Luer joint in the Plexiglas tube.

EVALUATION

Several parameters can be determined in the effluate, such as glucose for net uptake or lactate for metabolic formation. The values are plotted against time before and after addition of the test compounds, such as insulin or sulfonylureas.

CRITICAL ASSESSMENT OF THE METHOD

The isolated hindquarter of the rat has been proven to be a useful tool to study muscle metabolism under nearly physiological conditions after treatment with hormones and drugs as well as after electrical stimulation.

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K.6.3.2 Isolated diaphragm

See K.3.3.

K.6.3.3 Activity on isolated muscle cells

PURPOSE AND RATIONALE

Several types of cultured muscle cells haven been used in diabetes research:

BC_3H1 myocytes, the L6 cultured myogenic cell line, and rat cardiomyocytes.

The **BC_3H1 cell line** is a nonfusing spontaneously and reversibly differentiating mouse muscle cell line derived from a mouse neoplasm (Schubert et al. 1974). As for other cultured cell lines, the BC_3H1 myocytes demonstrate intermediate characteristics, possessing electron-microscopic features of both smooth and skeletal muscle but with a nicotinic acetylcholine receptor and an action potential more characteristic of skeletal muscle (Standaert et al. 1984).

The **L6 cultured myogenic cell line**, cultured from the thigh of a 1-day-old rat (Yaffe 1986) has many features of skeletal muscle (Pardridge et al. 1978).

Gorray et al. (1990) studied the antiproteolytic effects of glyburide by measuring the release of ^{14}C-tyrosine from intact L_6 myoblasts.

C_2C_{12} cells, a mouse skeletal muscle cell line has been isolated from dystrophic mouse muscle by Yaffe and Saxel (1977), McMahon et al. 1994; Ernst and White 1996).

Cardiomyocytes are isolated by perfusion of hearts from adult rats with collagenase (Eckel et al. 1983, 1991; Bähr et al. 1995).

Primary human muscle cell cultures were established by Sarabia et al. (1992).

PROCEDURE

BC_3H1 myocytes are cultured to confluence in 100 mm dishes over 10–14 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% Process Serum Replacement-I (Sigma, St. Louis, MO) and 25 mM glucose are added 18 h before the experiment. Cells are rinsed and pre-incubated at 37 °C for 20 min in Dulbecco's phosphate buffered saline with 0.1 mM $CaCl_2$ and 1 mg/ml BSA, then treated with buffer (controls) or agonists in buffer for 30 min.

Direct effects of sulfonylurea agents on glucose transport (Rogers et al. 1987), insulin-induced decrease in 5'-nucleotidase activity in skeletal muscle membranes (Klip et al. 1987), sulfonylurea-stimulated glucose transport association with diacylglycerol-like activation of protein kinase C (Cooper et al. 1990) were studied in this cell line.

L6 muscle cells are grown in 75-cm^2 flasks in Ham's F-10 medium containing 15% horse serum, 2.5 fetal calf serum, 0.87% glutamine, 0.87% penicillin-streptomycin, and 7.5% $NaHCO_3$ at 37 °C under a 5% CO_2 atmosphere. After 2–4 days, the cells are in a monolayer, confluent, aligned, and fused, but usually no myotubes are present. The medium is changed, and test substances are added.

Augmentation of the effects of insulin and insulin-like growth factors I and II on glucose uptake by sulfonylureas (Wang et al. 1987), coordinate regulation of glucose transporter function, and gene expression by insulin and sulfonylureas (Wang et al. 1989), glyburide-stimulated glucose transport via protein kinase C-mediated pathway (Davidson et al. 1991) were studied in this cell line.

C_2C_{12} cells, a mouse skeletal muscle cell line, have been shown to be suitable for stable transfection experiments of exogenous cDNA, making this cell line a candidate for stable transfections of cDNAs that encode mutant skeletal muscle and cardiac protein isoforms. The cells are available from CRL 1772 stock, American Type Culture collection. The cells are seeded into 100-mm diameter, collagen coated, tissue culture dishes at 5×10^4 cells/ml and plated in growth medium consisting of Dulbecco's modified Eagle's medium containing 20% fetal bovine serum, 0.5% chick embryo extract, and 1% penicillin/streptomycin. Cells are grown in this medium to between 75% and 85% confluence and then induced to differentiate by switching them to medium containing Dulbecco's modified Eagle's medium supplemented with 2% adult horse serum and 1% penicillin/streptomycin.

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K.6.4 Effects on adipose tissue

See K.3.2.

K.7 Inhibition of polysaccharide degrading enzymes

K.7.0.1 General considerations

Starch as the predominant ingredient of human food is rapidly degraded in the gastrointestinal tract by salivary and pancreatic α-amylaseα to maltose which is further hydrolyzed by maltase localized in the brush border of the small intestine to glucose. Glucose is immediately absorbed leading to hyperglycemia and consequently to hyperinsulinemia. Both phenomena are undesirable in diabetics and in obese patients. Inhibition of the digestion of starch leads to a decrease and a retardation of glucose absorption. In nature, α-amylase inhibitors are found in wheat and other grains (Shainkin and Birk 1970). Several inhibitors of amylase and α-glucosidase have been developed (Bischoff 1991). Animal experiments with high doses of absorbable α-glucosidase inhibitors indicate that lysosomal storage of glycogen may occur (Lembcke et al. 1991).

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K.7.0.2 Inhibition of α-amylase *in vitro*

PURPOSE AND RATIONALE

α-Amylase activity can be measured by determination of the reducing groups arising from hydrolysis of soluble starch by isolated pancreatic α-amylase. The reduction of 3,5-dinitrosalicylic acid to nitro-aminosalicylic acid produces a color shift which is followed photometrically by changes in the absorbance at 546 nm. Inhibition of starch hydrolysis by an α-amylase inhibitor results in a diminished absorbance at 546 nm in comparison with the controls.

PROCEDURE

Commercially available pancreatic α-amylase (E. Merck, Darmstadt, Germany) is used. Various concentrations of the α-amylase inhibitor are dissolved in 1 ml 20 mM Sörensen buffer, pH 6.9 and 10 mM NaCl. 0.1 ml pancreatic α-amylase solution in 0.4% BSA is added. After prior incubation at 25 °C, the enzymatic reaction is started by addition of 1.0 ml soluble starch solution. The reaction is stopped after 10 min with 1.0 ml of dinitrosalicylic acid reagent. The mixture is heated in a boiling water bath for 10 min, and after cooling measured at 546 nm against the reagent blank.

EVALUATION
Dose-response curves of enzyme inhibition are determined.

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K.7.0.3 Inhibition of glucosidase *in vitro*

PURPOSE AND RATIONALE
Inhibition of glucosidase can be measured *in vitro* using glucosidase from porcine small intestinal mucosa.

PROCEDURE
Glucosidase is prepared from rat or porcine small intestinal mucosa or porcine pancreas. The enzyme activity is assayed according to Dahlqvist (1964). The inhibitory activity is determined by incubating a solution (20 µl) of an enzyme preparation with 80 mM sodium phosphate buffer, pH 7.0 (500 µl) containing 37 mM sucrose or maltose, or 3.7 mM isomaltose and a solution (20 ml) containing various concentrations of the inhibitor at 37 °C for 20 min. The reaction mixture is heated for 2 min in a boiling water bath to stop the reaction. The amount of liberated glucose is measured by the glucose oxidase method.

EVALUATION
IC_{50} values of enzyme inhibition are determined.

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K.7.0.4 Evaluation of α-glucosidase inhibitors using the everted sac technique

PURPOSE AND RATIONALE
The everted sac technique allows to study the effects of intestinal enzymes on substrates in an incubation vial.

PROCEDURE
Male rats weighing 120–140 g are sacrificed and the small intestines removed by cutting across the upper end of the duodenal junction. The intestine is stripped of the mesentery and the entire intestinal content is rinsed with cold saline solution. The intestine is divided into 7 to 8 cm-segments and turned inside out using a Pasteur pipette. The everted intestine is ligated at one end with a cotton thread and a second ligature is placed loosely around the opposite end ready for tying. A 1 ml syringe with Krebs-Henseleit-buffer is introduced into the lumen sac. The end of the sac is ligated and placed in a 25 ml Erlenmeyer flask containing 6 ml of 1% starch, dissolved in Krebs-Henseleit-buffer, with or without various concentrations of the α-amylase inhibitor. Pork α-amylase (4 000 U/g starch) is also included in the 6 ml starch solution. Following gassing with 95% O_2/5% CO_2, the flask is tightly capped and incubated in a shaking bath at 37 °C for 120 min. The reaction is terminated by the addition of 10 µl 1 N HCl. At the end of the incubation period, the sac is removed from the flask and the inner fluid is collected by cutting one end of the sac. The final volumes of the solute in the serosal and the mucosal side and the level of glucose liberation are measured.

EVALUATION
Glucose liberated in the presence of various concentrations of the α-amylase inhibitor is expressed as percentage of glucose found without the inhibitor. Dose-response curves can be drawn plotting percent inhibition versus concentration of the inhibitor

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K.7.0.5 Inhibition of glucose absorption *in vivo*

PURPOSE AND RATIONALE
The inhibition of glucose absorption can be determined by measuring blood glucose after administration of starch or disaccharides with and without the inhibitor. In addition, non-absorbed starch or disaccharides can be determined in the intestine.

PROCEDURE
Male Wistar rats are kept on a standard diet with free access to tap water at constant temperature (24 ±1 °C). Sixteen hours prior to the experiment food but not wa-

ter is withheld. Groups of rats receive by stomach tube 2.5 g/kg raw starch in a water suspension without or with various doses of the α-amylase inhibitor. After 10, 20, 30, 60, 120 and 240 min, blood is withdrawn for determination of blood glucose and non-esterified fatty acids. The animals are sacrificed after these intervals and the residual starch in the stomach and the intestine determined. Definitely more starch is found in the intestine after simultaneous application of the α-amylase inhibitor. Similar experiments are performed in dogs for determination of serum insulin. The increase of blood glucose and serum insulin as well as the decrease of NEFA are inhibited.

EVALUATION

The values of starch content in stomach and intestine, as well as the blood glucose-, serum insulin- and NEFA-values are compared between control and treated animals.

MODIFICATIONS OF THE METHOD

In order to test the inhibition of glucosidase in addition, loading tests with sucrose or other disaccharides are performed (Puls et al. 1977; Matsuo et al. 1992).

Matsuo et al. (1992) performed experiments with genetically or experimentally obese rats (female Zucker fatty rats or female ventromedial hypothalamic nucleilesioned rats) and male yellow *KK*-mice.

LeMarchand-Brustel et al. (1990) studied the effect of an α-glycosidase inhibitor on experimentally induced obesity in mice. Male Swiss albino mice were rendered obese by injection of goldthioglucose at the age of 3 weeks. Since goldthioglucose treatment does not produce obesity in all injected mice, pro-obese mice were selected at 8 weeks of age on the basis of their body weight gain. They were divided in a control group receiving chow without drug and a treated group receiving chow containing the glycosidase inhibitor. Weight gain, glycemia and insulinemia were followed over 120 days.

Madar and Omusky (1991) studied the inhibition of intestinal α-glucosidase activity and postprandial hyperglycemia by α-glucosidase inhibitors in *fa/fa* rats. Various starch sources were used. Blood samples were taken at various intervals after starch load.

Takami et al. (1991) studied the antidiabetic actions of a disaccharidase inhibitor in spontaneously diabetic (GK) rats.

Okada et al. (1992) reported anti-obesity and antidiabetic actions of a new potent disaccharidase inhibitor in genetically obese-diabetic mice.

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K.8
Effect on secondary diabetes symptoms

K.8.1
Inhibition of aldose reductase

K.8.1.1
General considerations

Secondary symptoms of long lasting diabetes mellitus are diabetic neuropathy with sensory symptoms, motoric disturbances due to reduced nerve conduction velocity and diabetic cataracts. Both are related to enhanced conversion of glucose to polyols, such as sorbitol, by the enzyme aldose reductase (van Heyningen 1959; Clements 1979). Sorbitol is converted to fructose by sorbitol-dehydrogenase. A low activity of this enzyme enhances the accumulation of sorbitol, thus contributing to cellular damage. Inhibitors of aldose reductase have been developed with positive results in diabetic patients (Kador et al. 1985).

There are even some experimental hints for the benefit of aldose reductase inhibitors in diabetic cardiomyopathy (Cameron et al. 1989). Experimental data provide also evidence for possible beneficial effects in diabetic neuropathy, retinopathy, measured by nerve conduction velocity or electroretinography, lens cata-

ract formation, vascular permeability and filtration (Williamson et al. 1987; Tilton et al. 1989; Pugliese et al. 1990) and nephropathy (Sarges and Oates 1993).

Geisen et al. (1994) reported on sorbitol-accumulating pyrimidine derivatives which inhibited sorbitol dehydrogenase, induced a dose-dependent increase of tissue sorbitol and accelerated cataract development. Surprisingly, an acceleration of motor nerve conduction velocity in normal and diabetic rats and a normalization of glomerular filtration rates in diabetic rats were found.

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K.8.1.2 Aldose reductase inhibition *in vitro*

PURPOSE AND RATIONALE

The enzyme aldose reductase from bovine lens has been characterized by Hayman and Kinoshita (1965). Using this preparation, the *in vitro* effectiveness of various aldose reductase inhibitors can be determined (Varma and Kinoshita 1976; Billon et al. 1990).

PROCEDURE

Aldose reductase from lenses of calf eyes is isolated from the homogenates by ammonium sulfate precipitation of the concomitant proteins and by column chromatography on DEAE-cellulose. DL-Glyceraldehyde is used as substrate. The activity is expressed as the rate of OD_{340} [nm] due to the utilization of NADPH in the reaction:

$$\text{glyceraldehyde} + \text{NADPH} \xrightarrow{\text{aldose reductase}} \text{glycerol} + \text{NADP}$$

The reaction mixture contains: 0.1 M phosphate buffer, pH 6.2; NADPH 2.5×10^{-4} M; DL-glyceraldehyde 1.5×10^{-3} M; and the enzyme. The total volume of the reaction mixture is 1 ml. The reference blank consists of all the above compounds except the substrate. The effects of inhibitors on the enzyme activity are determined by including the compound being tested at the desired concentration in the reaction mixture. Appropriate blanks are used to correct for nonspecific reduction of NADPH and absorption by the compounds being tested. The test compounds are added first in 10^{-3} M solution and diluted as desired.

EVALUATION

Percentage of inhibition is tested at various concentrations and IC_{50}-values are calculated.

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K.8.1.3 Effect on nerve conduction velocity

PURPOSE AND RATIONALE

Aldose reductase inhibitors can be tested in rats with diabetes induced with streptozotocin by measuring nerve conduction velocity (Miyoshi and Goto 1973; Mayer and Tomlinson 1983; Tomlinson et al. 1882, 1984; Yue et al. 1982) and resistance to ischemic conduction block (Price et al. 1988).

PROCEDURE

Male Wistar rats weighing 100–150 g are rendered diabetic with an i.v. injection of 60 mg/kg streptozotocin. Diabetes is confirmed by the presence of glucosuria and elevated blood glucose levels 24 h after injection. The rats are kept on standard diet and water ad libitum for 28 days after streptozotocin treatment. Groups of rats are treated with the test compound (aldose reductase inhibitor) or the vehicle once daily by gavage starting 24 h after streptozotocin injection. One group of age-matched rats serves as control.

For measurement of sciatic **nerve conduction velocity**, rats are anesthetized with 2% halothane in oxygen and placed in a Perspex chamber that is maintained at a constant temperature by a copper coil in the base through which warm water is circulated. A fine needle thermocouple is inserted in the lateral aspect of the left hind limb, and the subcutaneous temperature is measured with an electronic thermometer. The conduction velocity of the left sciatic-tibial nerve is measured by a modification of the method of Sharma and Thomas (1974). Two fine platinum stimulating electrodes are inserted in the sciatic notch, and two more are inserted through the skin of the ankle to lie adjacently to the tibial nerve. The electrodes are 5 mm apart, the distal end being the cathode. A recording electrode is inserted under the skin of the lateral side of the foot and another between the third and fourth toes. A ground electrode is inserted under the skin on the dorsum of the foot. Both stimulation and recording are carried out with a neurophysiological unit (e.g., model MS92a, Medelec, Old Woking, Surrey, UK). The nerve is stimulated supramaximally with square-wave pulses of 0.1 ms duration. The conduction velocity is calculated as the distance between the distal electrodes divided by the difference between the latencies at the two stimulation points. The mean of three recordings is taken. The coefficient of variation of nerve conduction velocity is calculated by measuring nerve conduction velocity six times in one rat from each treatment group, withdrawing and reinserting the electrodes each time.

To measure **resistance to ischemic conduction block**, two fine platinum stimulating electrodes (1 cm apart) are inserted in the lateral aspect of the tail 3 cm from the base. Two recording electrodes are inserted near the tip, and a ground electrode is inserted midway between these and the stimulating electrodes. A thermocouple is attached to the middle of the tail. Both stimulation and recording are carried out as described above. To render the tail ischemic, a small sphygmomanometer cuff is inflated to 240 mm Hg around the base of the tail. The nerve is stimulated at 2.5 min intervals until the action potential disappears. The tail temperature is maintained at 32 °C. At the end of the experiment, 5 ml blood is taken by cardiac puncture. The animal is then sacrificed, the sciatic nerves are removed, cleaned, weighed, snap-frozen in liquid nitrogen and stored at –20 °C for subsequent estimation of polyol level.

Nerve polyol estimation is performed by the method of Stribling et al. (1985). Frozen nerves are thawed, placed in 1 ml water containing 60 µg of α-methylmannoside as an internal standard, and boiled for 20 min. Zinc sulfate (200 µl of a 5% solution) is added, samples are homogenized with a Polytron homogenizer, 200 µl 0.3 N barium hydroxide solution is added, and the samples rehomogenized. After centrifugation, the supernatant is freeze-dried overnight. The residue is silylated with 250 ml of a mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane (10 : 2 : 1; Pierce, Rockford, IL) at room temperature for 24 h. The reaction is stopped by adding 2 ml water, and 200 µl cyclohexane is added. Samples are shaken and 2 µl of the organic phase is injected into a gas chromatograph.

Plasma glucose levels are measured with the use of a glucose analyzer. Plasma fructosamine, which reflects plasma protein glycosylation, is measured with the use of a commercial kit.

Plasma glucose, plasma fructosamine and nerve sorbitol are increased and nerve conduction velocity decreased in streptozotocin treated rats.

EVALUATION

Results are expressed as means ±SE. Data from diabetic rats treated with the aldose-reductase inhibitor are compared with the values of untreated diabetic rats and normal controls. Statistical comparisons are made by use of the Mann-Whitney *U* test.

MODIFICATIONS OF THE METHOD

Sima et al. (1990) studied motor nerve conduction velocity and neuroanatomical abnormalities in insulin-deficient diabetic Bio-Breeding rats (BB-rat) with and without long-term administration of an aldose reduct-

ase inhibitor. Their findings of a significant, but incomplete prevention of neuropathy in these animals by aldose reductase inhibition suggest that additional mechanisms besides polyol-pathway activation may be of importance in the pathogenesis of diabetic neuropathy.

Carrington et al. (1991) studied effects on impulse conduction after aldose reductase inhibition with imirestat in sciatic nerves of streptozotocin-diabetic rats both *in vivo* and *in vitro*.

Cameron et al. (1991) studied nerve blood flow monitored by microelectrode polarography and hydrogen clearance in experimental diabetes in rats in relation to conduction deficits.

Schmidt et al. (1991) performed ultrastructural studies in mesenteric nerves of streptozotocin-induced diabetic rats.

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K.8.1.4 Effect on nerve blood flow (Doppler flux)

PURPOSE AND RATIONALE

In addition to nerve conduction studies, Calcutt et al. (1994) described a method to measure nerve blood flow by laser Doppler flowmetry in rats with streptozotocin induced diabetes treated with aldose reductase inhibitors.

PROCEDURE

Diabetes is induced in female Sprague-Dawley rats weighing 200–280 g by a single i.p. injection of 50 mg/kg streptozotocin after an overnight fast and is confirmed 2 days later by blood glucose determinations. Aldose-reduct-

ase inhibitor treated rats receive the test compound orally by gavage once a day for 2 months. For the experiment, the animals are anesthetized with an intraperitoneal injection (2 ml/kg) consisting of pentobarbital (12.5 mg/ml) and diazepam (1.25 mg/ml) in 0.9% NaCl. For estimation of nerve blood flow, a laser Doppler flowmeter (TSI model BPM 403A, St. Paul, MN) with a wavelength of 780 nm is used. Flow determinations are made with a filter frequency of 30 Hz to 18 kHz and, depending on the magnitude of the reflected signal, one of four band widths (30 Hz to 1.3 kHz, 30 Hz to 3 kHz, 30 Hz to 7.5 kHz, or 30 Hz to 18 kHz). The flow meter continuously displays a moving average for the preceding 5 s in units of $Hz \times 10^2$. For flow measurements, the sciatic nerve is exposed and the probe tip of the flowmeter placed 1 mm above the mid-thigh region of the nerve using a micromanipulator. After a 10 s stabilization period, the first measurement is recorded. The probe is then advanced 1 mm distally for a second measurement and this process repeated until 10 readings are recorded over 1 cm of nerve. The mean of 10 values is taken as average nerve laser Doppler flow. Blood flow velocity, expressed in m/s, is reduced in diabetic rats.

EVALUATION

The effects of streptozotocin diabetes and of aldose reductase treatment are assessed by one factor analysis of variance (ANOVA). Individual post-hoc comparisons are made with the Newman-Keuls method when the F ratio is $P < 0.05$.

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K.8.1.5 Electroretinogram in diabetic hyperglycemia and galactosemia

PURPOSE AND RATIONALE

Diabetic neuropathy is one of the important symptoms of long-lasting diabetes (Engerman 1989).

Several animal studies with aldose reductase inhibitors have been performed (Lightman et al. 1987; Hotta et al. 1988; Nagata and Robison 1988). Segawa et al. (1988) measured the development of electroretinogram abnormalities and the possible role of polyol pathway activity in diabetic hyperglycemia and galactosemia in the rat.

PROCEDURE

Male Sprague-Dawley rats weighing 310–400 g are dark-adapted for 20 min and anesthetized with i.p injections of ketamine, 50–80 mg/kg and atropine sulfate, 2 mg/kg. Electroretinography is performed monocularly with the pupil maximally dilated. Photic stimulation is delivered with an intensity of one J in 20 s interstimulus intervals. Using a contact lens-type electrode, electroretinograms (ERG) evoked by strong flushes are amplified by a preamplifier, displayed on an oscilloscope and summed using a signal averager which also provides a copy of the averaged ERG. The amplitudes of the a- and b-waves are measured from the base line to the trough of the a-wave and from the trough of the a-wave to the crest of the b-wave, respectively. The amplitudes of the oscillatory potentials are measured. The peak latencies are measured as the intervals between the stimulus onset and the peak of the corresponding a- and b-waves and oscillatory potentials. The oscillatory potentials (designated O_1, O_2, and O_3 in order of appearance on the ascending limb of the b-wave) are added together. The sum of these amplitudes is expressed as the wavelet index.

For inducing galactosemia, male Sprague-Dawley rats weighing 140–185 g at the beginning of the study, receive a diet of 30% galactose. Test compounds are given as 0.1% to the diet.

EVALUATION

Data are calculated as mean ±SEM and significance levels are estimated using the Wilcoxon rank sum test for unpaired data (two-sided). Linear regression is calculated by the least square method. A p-value of < 0.05 is regarded as statistically significant.

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K.8.1.6 Effect on streptozotocin induced cataract

PURPOSE AND RATIONALE

The increased incidence of cataracts, i.e., changes in the transparency or of the refractory index of the lens, in diabetic patients is well known. Evidence has accumulated for the involvement of polyol metabolism and the enzyme aldose reductase in diabetic cataractogenesis (van Heyningen 1959; Pirie and van Heyningen 1964). The enzyme aldose reductase catalyzes the reduction of aldoses such as glucose and galactose to the corresponding polyols, i.e., sorbitol and dulcitol. Sugar-induced cataractogenesis has been shown to parallel lenticular polyol accumulation (Kinoshita 1965). Since polyols do not readily diffuse through intact cellular membranes, they create a severe osmotic stress within the lenticular cells which leads to cellular swelling and loss of integrity of the cellular membrane (Kinoshita 1974). Aldose reductase inhibitors have been shown to prevent sugar induced cataracts (Dvornik et al. 1973; Varma and Kinoshita 1976; Kinoshita et al. 1979; Muller et al. 1985).

PROCEDURE

Experimental diabetes is induced in Wistar rats weighing 150–180 g by intravenous injection of 50 mg/kg streptozotocin (Griffin et al. 1984).

EVALUATION

The values of animals treated with the aldose reductase inhibitor are compared with the values of rats treated with streptozotocin only.

MODIFICATION OF THE METHOD

Instead of systemic application, the aldose reductase inhibitor is applied twice daily for 6 weeks in solution as eye drops to female Sprague-Dawley rats with a starting weight of 120–140 g after diabetes induction with 70 m/kg streptozotocin i.v. Body weight and blood sugar are registered twice a week. The progression of cataractous changes is followed by slit-lamp and Scheimpflug photography analysis every second week. At the end of the experiment, the animals are sacrificed and the lenses analyzed for their content of ATP, ADP, AMP, glucose, sorbitol, fructose, glucose-6-phosphate, and fructose-6-phosphate.

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K.8.1.7 Determination of aldose reductase in rat lens

PURPOSE AND RATIONALE

The effect of aldose reductase inhibitors can be tested by determining the level of aldose reductase, NADPH

and $NADP^+$ in the lenses of galactose treated or streptozotocin diabetic rats after treatment with the inhibitor compared to controls.

PROCEDURE

Cataract formation can be induced by galactose feeding to young rats. Male Sprague-Dawley rats weighing 40–50 g are randomly divided into 2 groups. One group is fed a laboratory chow, the other group is fed a galactose diet containing 50% galactose, 20% cornstarch, 15% casein, 9% hydrogenated oil, 4% salt mixture, and 2% cod liver oil.

Diabetes is induced in male Sprague-Dawley rats weighing 80–90 g by a single intravenous dose of 100 mg/kg streptozotocin.

The progression of cataracts is observed in controls and in rats treated with the aldose reductase inhibitor.

At appropriate times, the rats are sacrificed and the lenses are dissected. They are frozen on solid CO_2 and stored at –70 °C until analysis.

Single rat lenses are homogenized in 200 µl of 10 mM sodium phosphate buffer containing 5 mM 2-mercaptoethanol (pH 7.0) for 0.5 min at 0 °C. A cell-free extract is obtained by centrifuging the total lens homogenate at 17 300 *g* for 10 min. The enzyme reaction mixture (final volume 1 ml) contains 0.4 M $(NH_4)_2SO_4$, 0.1 M HEPES buffer adjusted to pH 7.0 with sodium hydroxide, 10 mM DL-glyceraldehyde and 0.12 mM NADPH. A 20 ml aliquot of supernatant is added to initiate the reaction. Decrease in absorbance at 340 nm is followed spectrophotometrically. Enzymatic activity is expressed as nM of NADPH oxidized per min.

EVALUATION

Results are expressed as aldose reductase units/mg lens (wet weight). Means ±SE are calculated and compared using Student's non-paired *t*-test.

MODIFICATIONS OF THE METHOD

Naeser et al. (1988) studied sorbitol metabolism in the retina, optic nerve, and sural nerve of diabetic **rats** treated with an aldose reductase inhibitor. Activities of aldose reductase, sorbitol-dehydrogenase, and content of sorbitol were assayed in these tissues.

Freeze-dried tissue samples are sonicated for 5–10 s in 215 µl 0.04 M Tris(hydroxymethyl)-aminomethane, pH 6.8. For determination of aldose reductase, the homogenate is centrifuged for 45 min at 37 000 rpm and 100 µl is taken from the supernatant for analysis. The remaining sample is centrifuged further for 2 h at 96 000 rpm and the supernatant used for sorbitol determination. For determination of aldose reductase, 10 µl of the sample are mixed with 15 µl buffer solution containing 0.14 mol/L Tris(hydroxymethl)-aminomethane, 151 µmol/L NADH, and 0.6 mol/L glucose. After incubation in a water bath for 20 min at 38 °C, the incubation is stopped by transfer to ice. Samples mixed with buffer solution without glucose serve as blanks. Twenty µl of the incubated material are then mixed with 1.25 ml 0.04 mol/L NaOH solution containing 1.7 mmol/L NaCl. Fluorescence is measured in a Farrand Ratio Fluorometer with primary filters 5 860 and 5 970 (Corning Glass Works) and secondary filters 2A (Turner Optical Co), 4 308 and 3 387 (Corning Glass Works). The amount of NADPH consumed serves as measure for aldose reductase activity.

Sorbitol-dehydrogenase is measured by adding 5 µl of fresh homogenate to 20 µl of a buffer composed of 0.1 mol/L Tris(hydroxymethyl)-aminomethane, 1.25 mmol/L NAD and 50 mmol/L sorbitol at pH 6.8. After incubation for 30 min at 38 °C, the reaction is stopped by the addition of 5 µl 0.1 N NaOH and transfer to ice. The amount of NAD^+ converted to NADH is determined luminometrically in 10 µl samples with 100 µl of NAD(P)H reagent (Boehringer Mannheim, Germany) containing 5 mg/L luciferase, 25 mmol/L potassium phosphate, 100 mmol/L dithiothreitol, 38 U/L Triton-X 100, and 20 mmol/L myristic aldehyde. The flux of light is instantly registered, using a photomultiplier.

Sorbitol is determined in the centrifugation supernatant. After immersion in boiling water for 5 min, aliquots of 15 µl are added to 10 µl buffer containing 0.1 mol/L Tris, 3.5 mmol/L NAD, and 2.7 U/ml sorbitol-dehydrogenase at pH 8.6. Tissue blanks are run without sorbitol-dehydrogenase. The incubation is terminated and the amount of NADH formed is determined as for the sorbitol-dehydrogenase assay.

Gonzales et al. (1986) studied the effect of an aldose reductase inhibitor on integration of polyol pathway, pentose phosphate pathway and glycolytic route in diabetic rat lens. Diabetes was induced in male Wistar rats weighing about 230 g by subcutaneous injection of 200 mg/kg alloxan monohydrate. After one week, in the diabetic rat lens was an apparent increase in the flux of glucose through the pentose phosphate pathway, as measured by the difference in the yields of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose [C1–C6]. Treatment with the aldose reductase inhibitor reduced the values toward normal. With glucose tritiated on carbons 2 or 3, it has been shown that the flux of glucose through the polyol route is increased, whereas the flux through the glycolytic pathway is decreased in the diabetic rat lens. Both parameters were restored to normal in diabetic rats treated with the aldose reductase inhibitor.

Meydani et al. (1994) investigated the onset and progression of cataract in weanling Sprague Dawley rats fed 10, 15, 20 and 30% dietary galactose for 45–226 days.

Ohta et al. (1999) studied cataract development in 12-months-old rats fed a 25% galactose diet and its relation to osmotic stress and oxidative damage.

Using phosphorus-31 nuclear magnetic resonance spectroscopy, Sakagami et al. (1999) investigated the metabolic kinetics of organophosphate compounds in the rat lens during cataract development induced by different doses of galactose added to rat chow.

Mackic et al. (1994) developed a model of galactose-induced cataract formation in **guinea pigs** and studied the morphologic changes and accumulation of galactitol.

Sato et al. (1998) demonstrated that the formation and progression sugar cataracts in galactose-fed **dogs** can be dose-dependently inhibited by the aldose reductase inhibitors.

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K.8.1.8 Effect on naphthalene induced cataract

PURPOSE AND RATIONALE

Systemic application of naphthalene induces cataract in Brown-Norway rats. Aldose reductase inhibitors are tested for prevention of the cataract changes by parallel systemic or topical application (as eye drops).

PROCEDURE

Female Brown-Norway rats at an age of 6–7 weeks with a starting weight of 70–100 g are used. They are fed standard lab chow and receive water ad libitum. Naphthalene is dissolved in warm paraffin oil (10 g/100 ml) and is administered orally by gavage every second day in a dose of 1 g/kg. The test com-pound is administered orally every day or applied topically as suspension every day once, twice or four times to the right eye. The duration of the treatment is 6 weeks. Slit lamp microscopy (Zeiss photo slit lamp) during mydriasis with 1% atropine eye drops is performed in weekly intervals, including a baseline examination before the start of the treatment and a final examination prior to sacrifice. Scheimpflug photography of the anterior eye segment (Topcon SL-45 Scheimpflug camera) is carried out at the baseline examination, after 3 weeks of treatment and at the end of the experiment. Prior to sacrifice blood samples are taken by cardiac puncture under ether anesthesia. After sacrifice, lens fresh weight, concentration of oxidized and reduced glutathione, and the concentration of the aldose reductase inhibitor in the lenses and in blood samples are determined.

EVALUATION

The values of animals treated with the aldose reductase inhibitor are compared with the values of rats treated with naphthalene only.

MODIFICATIONS OF THE METHOD

Rathbun et al. (1996a) induced rapid-onset cataracts in SPF C57 bl/6 mice by i.p. administration of naphtha-

lene following cytochrome P-450 induction with phenobarbital.

Rathbun et al. (1996b) assessed the activity of a L-cysteine prodrug suitable for glutathione biosynthesis rat lenses *in vitro* and as an agent for the prevention of acetaminophen- and naphthalene-induced murine cataract in genetically susceptible mice.

Holmen et al. (1999) compared different methods of photographic evaluation of cataract formation in rats in response to different regimes of naphthalene treatment.

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Chapter L
Anti-obesity activity[1]

[1] Review by M. Bickel, contributions by A. W. Herling.

L.1 Methods to induce experimental obesity

L.1.0.1 General considerations

Influence of the central nervous system, in particular of the hypothalamus, on development of obesity has been suspected since the early clinical observations of Babinski (1900) and Fröhlich (1901), Biedl 1916). Experiments reported by Smith (1927, 1930) showed that injections of chromic acid into the suprasellular region of rats with lesion of the hypothalamus induced obesity in rats (Bomskov 1939). Hetherington and Ranson (1939) found that electrolytic lesions, restricted to the ventromedial region of the hypothalamus, could be associated with the development of obesity.

A virally induced obesity syndrome in mice was described by Lyons et al. (1982).

Chan (1995) gave a review on β-cell stimulus-secretion coupling defects in rodent models of obesity.

Leiter and Herberg (1997) reviewed the advances in understanding the molecular bases for monogenic obesity mutations capable of producing obesity-induced diabetes, or diabesity in mice.

Astrup and Lundsgaard (1998) discussed pharmacological mechanisms of anti-obesity drugs.

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L.1.0.2 Food induced obesity

PURPOSE AND RATIONALE

Obesity can be induced in rats by offering a diet containing corn oil and condensed milk.

PROCEDURE

Male Sprague Dawley rats are housed in individual wire-bottom suspended cages in rooms maintained at 22–23 °C with 12 h light-dark cycles. At the age of 6 months (body weight about 450 g) the animals are divided in 2 groups: group I is fed ordinary Purina Rodent Chow, group II a special diet containing Purina Rodent Chow, corn oil and condensed milk, resulting in a composition of 14.7% protein, 44.2% carbohydrate, 15.8% lipid, 2.5% fiber, 1.2% vitamin mixture, and 19% water. Body weight and food intakes are measured, and diet replaced, every 3 to 4 days.

Three months from the start of the experiment, the rats are sacrificed by decapitation for determination of adipose tissue cell size and number, carcass composition and plasma lipids, and hormone and glucose levels.

The dorsal subcutaneous (inguinal) pad, the retroperitoneal pad, and one epididymal fat pad are sampled for determination of lipid content by the method of Folch et al. (1957). The method consists of homogenizing the tissue with a 2 : 1 chloroform-methanol mixture and washing the extract by addition to it of 0.2 its volume of water. The resulting mixture separates into two phases. The lower phase is the total pure lipid extract. Cell number in each pad is determined by the osmium fixation method of Hirsch and Gallian (1968) using a Coulter counter. Total epididymal pad weights are based on doubling the weight of the individual pads sampled.

EVALUATION

Intergroup comparisons are made with the use of a two-tailed Student's *t*-test.

MODIFICATIONS OF THE METHOD

Scalfani and Springer (1976) induced obesity in adult female rats by adding a variety of supermarket foods to lab chow (**"cafeteria diet"**). In behavioral tests, the authors found similarities to hypothalamic and human obesity syndromes.

Rothwell et al. (1982) compared the effects of feeding a cafeteria diet on energy balance and diet-induced thermogenesis in four strains of rats.

Stock and Rothwell (1979) discussed the influence of various forms of feeding, high-fat diets, insulin injections, tube-feeding, and cafeteria feeding on energy balance in laboratory animals.

Rolls et al. (1980) found persistent obesity in rats following a period of consumption of a mixed, high energy diet. When the high energy foods were withdrawn after 90 days and just chow was available, the obese rats maintained their elevated body weights.

Hill et al. (1992) studied the influence of amount and composition of dietary fat on development of obesity in rats. Adult male Wistar rats were fed high fat (HF; 60% of calories) or low fat (LF; 20% of calories) diets for 28 weeks. Half of the rats in each condition received diets with saturated fat (lard) or with polyunsaturated fat (corn oil). There was some indication that unsaturated fat diets were associated with greater accumulation of fat in subcutaneous tissue depots than saturated fat diets. The effects of the type of fat were less than those of the amount of dietary fat.

Wade and Gray (1979) reviewed the gonadal effects on food intake and adiposity in rats. Estradiol and testosterone decrease adiposity, while progesterone increases carcass fat content.

Thermogenesis induced by various diets has been discussed by Rothwell and Stock (1986).

Harris (1993) studied the impact of high- or low-fat cafeteria foods on nutrient intake and growth of rats consuming a diet containing 30% energy as fat.

Segues et al. (1994) studied long-term effects of cafeteria diet feeding on young female Wistar rats by comparing the circulating levels of glucose, lactate, glycerol, 3-hydroxybutyrate and urea, and liver glycogen.

Llado et al. (1997) studied fatty acid composition of brown adipose tissue in dietary obese rats. Long time exposure to a hypercaloric high-fat diet such as the cafeteria diet induced an important fatty acid accumulation in brown adipose tissue, mainly for the major saturated and monounsaturated fatty acids.

LeBlanc and Labrie (1997) investigated the role of palatability of the food in diet-induced thermogenesis.

Selective breeding for diet-induced obesity and resistance in Sprague-Dawley rats was reported by Levin et al. (1997).

Herberg et al. (1974) reported the effects of either a high-carbohydrate diet or a high-fat diet over prolonged periods in metabolically intact and in obese NZO **mice**.

West et al. (1992) evaluated the effects of a 7 week consumption of a diet containing 32.6% of kilocalories as fat (condensed milk) on body composition and energy intake in nine strains of inbred mice. Relative to Chow diet controls, the condensed milk diet significantly increased carcass lipid content in six strains (AKR/J, C57L/J, A/J, C3H/HeJ, DBA/2J, and C57BL/6J), but had no or a marginal effect on adiposity in 3 strains of mice (SJL/J, I/STN, and SWR/J).

West et al. (1994) studied the genetics of dietary obesity in AKR/J x RWR/J mice. Pups were weaned between the ages of 29 and 34 days onto Purina Chow ad libitum until they were switched to a high fat, condensed milk diet containing 32.6%, 15.0%, and 52.4% of kilocalories as fat, protein, and carbohydrate, respectively.

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L.1.0.3 Hypothalamic obesity

PURPOSE AND RATIONALE

Hyperphagia in rats has been reported after hypothalamic lesions (Liu and Yin 1974). Surgical techniques were described by Leibowitz et al. (1981), Vander Tuig et al. (1985).

PROCEDURE

Female Sprague Dawley rats, weighing about 190 g body weight, receive during a 5–9 days initial period of adjustment a high-fat diet. They are then fasted overnight, anesthetized with 35 mg/kg pentobarbital sodium and additionally 1 mg atropine methyl nitrate intraperitoneally. Bilateral wire knife cuts or electrolytic lesions are sterotaxically positioned in the hypothalamus (David Kopf Instruments, CA). With the incision bar positioned at –3.0 mm, parasagittal wire knife cuts are placed between the medial and lateral hypothalamus using a retractable wire knife according to Gold et al. (1973). The cuts are made 1.0 mm lateral to the midline and extended from 8.5 to 5.5 mm anterior to the ear bars and from the base of the brain dorsally 3.0 mm.

To produce electrolytic lesions in the ventromedial hypothalamus, the incision bar is positioned at +5.0 mm and a stainless steel electrode, insulated except for 0.5 mm at the tip, is lowered 0.6 mm lateral to the midline and 5.8 mm anterior to the ear bars. With the tip of the electrode 0.7 mm above the base of the brain, lesions are made by passing 2.0 mA of anodal current for 20 s to a rectal electrode.

Sham-operated rats serve as controls. Separate rats that are fasted overnight are killed at the time of surgery to provide data on initial body composition.

Histological verification of placement of knife cuts and lesions is made in brains fixed in 10% buffered formaldehyde solution and embedded in paraffin. Serial sections through the hypothalamic area of the brain are examined histologically.

EVALUATION

Not only food consumption and increase of body weight can be determined after hypothalamic lesions, but also brown adipose tissue enzymes and guanosine diphosphate binding to brown adipose tissue mitochondria and noradrenaline turnover in various organs.

MODIFICATIONS OF THE METHOD

A survey on hypothalamic and genetic obesity in experimental animals was given by Bray and York (1979).

Caloric compensation to gastric loads in rats with hypothalamic hyperphagia was reported by Liu and Yin (1974).

Sclafani and Aravich (1983) adapted adult female Sprague Dawley rats to a macronutrient self-selection regimen which allowed them ad libitum access to separate sources of protein, carbohydrate, and fat. The rats were then given either ventromedial hypothalamic lesions, paraventricular hypothalamic lesions, parasagittal knife cuts through medial hypothalamus or sham lesions. Following surgery, all lesioned rats overate and became obese as compared to sham operated controls. The group with parasagittal knife cuts through medial hypothalamus gained more weight than the groups with ventromedial hypothalamic lesions and paraventricular hypothalamic lesions.

Enhanced expression of rat obese (ob) gene in adipose tissue of ventromedial hypothalamus (VMH)-lesioned rats was described by Funahashi et al. (1995).

Elmquist et al. (1999) discussed the role of leptin and other peptide hormones in different areas of the hypothalamus in controlling food intake and body weight.

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L.1.0.4 Goldthioglucose-induced obesity

PURPOSE AND RATIONALE

Intraperitoneal or intramuscular injection of goldthioglucose induces obesity in mice. The effect is related to destruction of hypothalamic and extra-hypothalamic areas of the brain (Perry and Liebelt 1961).

PROCEDURE

Swiss albino mice of either sex are fed with commercial mouse chow (Altromin R®) ad libitum. At the age of 6 weeks, the animals receive a single intraperitoneal injection of 30–40 mg/kg gold-thioglucose.

EVALUATION

Food intake is registered for 2 weeks and body weight for a period of 3 months and compared with untreated controls.

MODIFICATIONS OF THE METHOD

Debons et al. (1962, 1968, 1977) administered 800 mg/kg gold thioglucose by a single intraperitoneal injection to female CBA mice.

Obesity was induced in rats by implants of gold thioglucose in the hypothalamus (Smith and Britt 1971; Smith 1972).

Several other compounds produce obesity concomitantly with brain lesions.

Single intraperitoneal injection of bipiperidyl mustard (N,N'-bis-[β-chloroethyl]-4,4'-bipiperidine) in doses between 5 to 50 mg/kg induced obesity in mice (Rutman et al. 1966). Brain lesions and obesity by bipiperidyl mustard could also produced in rats (Laughton and Powley 1981).

Massive and persistent obesity was induced in mice by a single intracerebral injection with 4-nitroquinoline 1-oxide (Mizutani 1977).

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L.1.0.5 Monosodium glutamate-induced obesity

PURPOSE AND RATIONALE

Adiposity can be induced in mice by repeated subcutaneous injections of monosodium-L-glutamate at an early stage of life (Olney 1969).

PROCEDURE

Male Charles River mice are treated immediately after birth with daily subcutaneous injections of 2 g/kg monosodium-L-glutamate for 5 consecutive days. Control mice are treated with physiological saline. The animals are weaned at 3 weeks of age, housed under controlled temperature and artificial light/dark cycle and provided with commercial powdered chow and tap water at libitum.

EVALUATION

Food consumption and weight gain is measured at weekly intervals.

MODIFICATIONS OF THE MEDTHOD

Tokuyama and Himms-Hagen (1986) studied brown adipose tissue thermogenesis, torpor, and obesity in glutamate-treated C57B1/6J mice offered either normal chow or a cafeteria diet and found that the high metabolic efficiency and obesity of the glutamate-obese animals are principally a consequence of their maintenance of a hypothermic torpid state for more than 50% of the time.

Seress (1982) injected young albino **rats** with either single or repeated doses of 0.1–6.0 mg/g body weight of monosodium L-glutamate between the ages of 2 and 40 days. The smallest, single effective dose was 0.25 mg/g body weight administered during the first week of life. The sensitivity to monosodium L-glutamate decreased with age. In adult rats an 80–90% loss of neurons in the anterior part of the arcuate nucleus was found.

Remke et al. (1988) reported on development of hypothalamic obesity after subcutaneous administration of monosodium glutamate to neonate rats.

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L.2 Genetically obese animals

L.2.0.1 General considerations

Obesity and diabetes are syndromes quite often linked in patients (maturity onset diabetes) and hereditary animal models. Hunt et al. (1976) described animal models of diabetes and obesity including the PBB-Ld mouse. A survey on hypothalamic and genetic obesity in experimental animals was given by Bray and York (1979). The inheritance of obesity in animal models of obesity was discussed by Festing (1979). Cawthorne (1979) discussed the use of animal models in the detection and evaluation of compounds for the treatment of obesity. The regulation of body weight in animals by leptin was reviewed by Friedman and Halaas (1998).

Symptoms of diabetes and obesity are overlapping in many animal models (see also K.2: Genetically diabetic animals).

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L.2.0.2 Spontaneously obese rats

The occurrence of spontaneous obesity has been reported in several strains of rats:

WBN/KOB RAT

Spontaneous hyperglycemia, glucosuria and glucose intolerance have been observed in aged males of an inbred Wistar strain, named the WBN/Kob rat (Nakama et al. 1985; Tsuchitani et al. 1985; Koizumi 1989). These animals exhibit impaired glucose tolerance and glucosuria at 21 weeks of age. Obvious decreases in the number and size of islets are found already after 12 weeks of age. Fibrinous exudation and degeneration of pancreatic tissue are observed in the exocrine part, mainly around degenerated islets and pancreatic ducts in 16 weeks old males.

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ZUCKER-FATTY RAT

The Zucker-fatty rat is a classic model of hyperinsulinemic obesity. (Zucker 1965). Obesity is due to a simple autosomal recessive (*fa*) gene and develops at an early age. Obese Zucker rats manifest mild glucose intolerance, hyperinsulinemia, and peripheral insulin resistance similar to human NIDDM. However, their blood sugar level is usually normal throughout life (Bray 1977; Clark et al. 1983; McCaleb and Sredy 1992; Abadie et al. 1993; Alamzadeh et al. 1993; Kasim et al. 1993; Galante et al. 1994).

Truett et al. (1991) found evidence that the rat obesity gene fatty (*fa*) has homology with the mouse gene diabetes (*db*).

Triscari and Sullivan (1987) reported a normalizing effect of an inhibitor of thromboxane synthase on the hyperinsulinemic state of obese Zucker rats and diet-induced obese rats.

Rouru et al. (1993) described the effect of chronic treatment with a 5-HT_1 receptor agonist on food intake, weight gain, plasma insulin and neuropeptide Y mRNA expression in obese Zucker rats.

Santti et al. (1994) studied the potentiation of the anti-obesity effect of a β_3-adrenoceptor agonist in obese Zucker rats by exercise.

Savontaus et al. (1997) investigated the anti-obesity effect of a imidazoline derivative in genetically obese (fa/fa) Zucker rats.

Zhang et al. (1996) reported downregulation of the expression of the *obese* gene by an antidiabetic thiazolidinedione in Zucker diabetic fatty rats and *db/db* mice.

Lynch et al. (1992) identified several adipocyte proteins, among them pyruvate decarboxylase contributing to the increased lipogenic capacity of young obese Zucker adipocytes.

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WDF/TA-FA RAT

The WDF/Ta-fa rat, commonly referred to as the Wistar fatty rat, is a genetically obese, hyperglycemic rat established by the transfer of the fatty (*fa*) gene from the Zucker rat to the Wistar Kyoto rat. (Ikeda et al. 1981; Kava et al. 1989; Velasquez et al. 1990). The Wistar fatty

rat exhibits obesity, hyperinsulinemia, glucose intolerance, hyperlipidemia, and hyperphagia similar to Zucker rats being, however, more glucose intolerant and insulin resistant than Zucker rats. Hyperglycemia is usually not observed in females but can be induced by addition of sucrose to the diet.

Kobayashi et al. (1992) found an increase of insulin sensitivity by activation of insulin receptor kinase by Pioglitazone in Wistar fatty rats (*fa/fa*).

Mazusaki et al. (1996) found an augmented expression of the *obese* (*ob*) gene during the process of obesity in genetically obese-hyperglycemic Wistar fatty (*fa/fa*) rats.

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OLETF RAT

A spontaneously diabetic rat with polyuria, polydipsia, and mild obesity was discovered in 1984 in an outbred colony of Long-Evans rats. A strain of rats developed from this rat by selective breeding has since been maintained at the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) and named OLETF. The characteristic features of OLETF rats are: (1) late onset of hyperglycemia (after 18 weeks of age), (2) a chronic course of disease, (3) mild obesity, (4) inheritance by males, (5) hyperplastic foci of pancreatic islets, and (6) renal complications (nodular lesions). The clinical and pathological features of disease in OLETF rats resemble those of human NIDDM.

Administration of diazoxide (0.2% in diet), an inhibitor of insulin secretion, to OLETF rats from the age of 4 to 12 weeks completely prevented the development of obesity and insulin resistance (Aizawa et al. 1995).

Ishida et al. (1995) found that insulin resistance preceded impaired insulin secretion in OLETF rats.

Umekawa et al. (1997) determined induction of uncoupling protein and activation of GLUT4 in OLETF rats after administration of a specific β_3-adrenoceptor agonist.

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OBESE SHR RAT

The strain of obese SHR rats was developed by Koletsky (1973, 1975) by mating a spontaneous hypertensive female rat of the Kyoto-Wistar strain with a normotensive Sprague Dawley male. After several generations of selective inbreeding, these obese SHR exhibited obesity, hypertension, and hyperlipidemia. In addition, some rats developed hyperglycemia and glucosuria associated with giant hyperplasia of pancreatic islets.

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JCR:LA-CORPULENT RAT

Several substrains were developed from obese SHR rats, such as the JCR:LA-corpulent rat which exhibits a syndrome characterized by obesity, hypertriglyceridemia and hyperinsulinemia with impaired glucose tolerance and is susceptible to vascular arteriosclerotic lesions (Russell et al. 1986a,b, 1994).

Compared to fatty Zucker rats, the JCR:LA-corpulent rats have higher levels of the insulin releasing hormone gastric inhibitory polypeptide and higher insulin levels (Pederson et al. 1991).

Vydelingum et al. (1995) found an overexpression of the obese gene in the JCR:LA-corpulent rat.

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GROWTH HORMONE-DEFICIENT DWARF RAT

Clark et al. (1996) described the obese growth hormone-deficient dwarf rat as a new model of obesity.

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L.2.0.3 Spontaneously obese mice

YELLOW OBESE (A^YA) MOUSE

The yellow obese mouse in the only example of obesity inherited through a dominant gene and was described as early as 1883 by Lataste and in 1905 by Cuenot. It is located on chromosome 2 at linkage group 5, the agouti locus (Bateson 1903). Since the genes controlling obesity and the agouti coat colors are so closely linked, the obesity is associated with a change of pigmentation from black to yellow. Such an association allows the early identification of pre-obese mice as soon as the coat hair begins to grow.

Since the original description of the yellow (A^ya) mouse, a number of additional alleles have appeared at the agouti locus. The homozygous dominant yellow mutation (A^y/A^y) is lethal in utero (Robertson 1942; Eaton and Green 1962) with approximately 25% of any litter from A^ya matings dying from an abnormal development after the trophoblast stage (Pedersen 1974).

Yellow (A^ya) mice develop a moderate form of obesity and diabetes. Increased body weight first appears at the time of puberty (8–12 wk) (Dickie and Wooley 1946; Carpenter and Mayer 1958), after which body weight increases slowly to reach values of approximately 40 g. In contrast to other forms of obesity, yellow mice are characterized by increased linear growth. Plasma insulin concentrations are increased and food is stored more efficiently than in lean mice (Dickerson and Gowan 1967). Food intake returns to normal in older A^ya mice and the animals lose body weight (Hollifield and Parson 1957). The obesity may be exaggerated by being fed high-fat diets (Fenton and Chase 1951; Silberberg and Silberberg 1957; Carpenter and Mayer 1958). Food restriction may normalize body weight but the animals still remain obese (Fenton and Chase 1951; Hollifield and Parson 1957). Metabolic rate of A^ya mice is depressed when related to body surface, although oxygen consumption per animal is identical to the homozygous recessive agouti (a/a) mouse (Bartke and Gorecki 1968).

Gill and Yen (1991) studied the effect of ciglitazone on endogenous plasma islet amyloid polypeptide (amylin) and insulin sensitivity in obese-diabetic viable yellow mice (VY/Wfl-A^{vy}/a).

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KK-A^Y MOUSE

Iwatsuka et al. (1970) reported on yellow KK mice (also named KK-A^y mice), carrying the yellow obese gene (A^y). These mice develop marked adiposity and diabetic symptoms in comparison with their littermates, black KK mice. Blood glucose and circulating insulin levels as well as HbA_{1c} levels were increased progressively from 5 weeks of age. Degranulation and glycogen infiltration of B cells were followed by hypertrophy and central cavitation of islets. Lipogenesis by liver and adipose tissue were increased. Insulin sensitivity of adipose tissue was more remarkably reduced than in black KK mice to its complete loss at 16 weeks of age. Renal involvement is uniquely marked by early onset and rapid development of glomerular basement membrane thickening (Diani et al. 1987).

KK-A^y mice can be used to demonstrate the extrapancreatic action of antidiabetic drugs, such as glimepiride, a novel sulfonylurea (Satoh et al. 1994).

Sohda et al. (1990) evaluated ciglitazone and a series of 5-[4-(pyridylalkoxy)benzyl]-2,4-thiazolidinediones for hypoglycemic and hypolipemic activities in yellow KK mice.

Hofmann et al. (1992) evaluated the expression of the liver glucose transporter GLUT2 and the activity and the expression of phosphoenolpyruvate carboxykinase in the liver of obese KKA^Y mice after treatment with the oral antidiabetic agent pioglitazone.

Yoshida et al. (1991) compared brown adipose tissue thermogenesis, resting metabolic rate, insulin receptors in adipocytes, and blood glucose and serum insulin levels during a glucose overloading test in yellow KK mice with C57B1 control mice after a β_3-adrenoceptor agonist.

Yoshida et al. (1996) determined body weight, food intake, white adipose tissue weight, brown adipose tissue weight and its thermogenesis, noradrenaline turnover, blood glucose and serum insulin levels and GLUT4 in diabetic yellow KK mice compared with C57B1 mice after mazindol.

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OBESE HYPERGLYCEMIC (OB/OB) MICE

Ingalls et al. (1950), Mayer et al. (1951), Bleisch et al. (1952) observed hereditary diabetes in genetically obese mice. The obese hyperglycemic mice were glycosuric, the non-fasting blood sugar levels were about 300 mg%, but neither ketonuria nor coma were observed. One of the most interesting features was insulin-resistance; doses as high as 400 IU/kg had little effect on blood sugar. The serum insulin-like activity was high, the islands of Langerhans were hypertrophic, their insulin content was increased and the liver glycogen stores were decreased. Kidneys and other organs did not show pathological changes. Obviously, the diabetic condition of this and other strains of obese hyperglycemic mice is different from that of the human diabetic patient.

Reduced oxygen consumption has been noted as early as 10–18 days of age in *ob/ob* mice (Boissenault et al. 1976; Trayhurn 1977).

Other strains or substrains of mice with obesity and hyperglycemia have been described by Dickie (1962), Westman (1968), Stein et al. (1970), Coleman and Hummel (1973), Herberg and Coleman (1977).

Strautz (1970) implanted *ob/ob* mice with Millipore diffusion chambers containing islets isolated from pancreas of normal littermates.

Trayhurn et al. (1977) found a thermogenic defect in pre-obese *ob/ob* mice. Rectal temperature of 17 days old pre-obese mice in response to an environmental temperature of 4 °C fell much more than in lean controls.

Chlouverakis (1972) performed parabiotic experiments of obese-hyperglycemic mice (*ob/ob*) with lean littermates and determined body weight, glucose, serum insulin and triglycerides as well as insulin-sensitivity of diaphragm muscle and epididymal fat pad.

Parabiosis of obese (*ob/ob*) with diabetes (*db/db*) mice caused the obese partner to become hypoglycemic, to lose weight and to die of starvation, while no abnormal changes were observed in the diabetic partner (Coleman 1973).

Cresto et al. (1977) compared the rate of insulin degradation in normal and in *ob/ob* mice.

Zhang et al. (1994) succeeded in positional cloning of the mouse *obese* gene and its human homologue.

Pelleymounter et al. (1995) investigated the effects of the *obese* gene product on body weight regulation in *ob/ob* mice. The OB protein was expressed in *E. coli* and purified to homogeneity as a 16-kilodalton monomer. Daily intraperitoneal injections of the recombinant OB protein to *ob/ob* mice lowered their body weight, percent body fat, food intake, and serum concentrations of glucose and insulin.

Halaas et al. (1995) reported that daily intraperitoneal injections of either mouse or human recombinant OB protein reduced the body weight of *ob/ob* mice but had no effect on *db/db* mice.

Campfield et al. (1995) found that peripheral and central administration of microgram doses of recombinant mouse OB protein reduced food intake and body weight of *ob/ob* and diet-induced obese mice but not in *db/db* obese mice.

Trayhurn et al. (1996) studied the effects of fasting and refeeding on *ob* gene expression in white adipose tissue of lean and obese *(ob/ob)* mice using a 33-mer antisense oligonucleotide as a probe for the rapid chemiluminescence-based detection of *ob* mRNA.

Sterility defect in homozygous obese female mice could be corrected by treatment with the human recombinant OB protein leptin (Chehab et al. 1996).

Roupas et al. (1990) used isolated adipocytes from *ob/ob* mice to study the diabetogenic action of growth hormone.

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BL/6 OBESE MICE

An obese mutation occurred in a noninbred stock (Ingalls et al. 1950) but was established later, and has been maintained, in the C5BL/6J (BL/6) strain. BL/6 obese mice are characterized by marked obesity, hyperphagia, transient hyperglycemia and markedly elevated plasma insulin concentrations associated with an increase in number and size of beta cells in the islets of Langerhans (Coleman and Hummel 1973; Genuth et al. 1971). The mutation is autosomal recessive and homozygous mutants of both sexes are infertile. Obese mutants are obtained by mating known heterozygotes. A primary metabolic disturbance in the adipocyte has been postulated since increased adipocyte size has been observed as early as 14 days of age well before any obesity or hyperinsulinemia are observed (Joosten and van der Kroon 1974).

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NZO MOUSE

The New Zealand obese (NZO) mouse was first described in 1953 by Bielschowsky and Bielschowsky. The strain was developed by selective inbreeding of obese mice from a mixed colony, beginning from a pair of agouti mice, which also gave rise to the NZB black strain (Melez et al. 1980). NZO mice develop obesity, mild hyperglycemia, glucose intolerance, hyperinsulinemia, and insulin resistance. The adult NZO mouse normally attains a body weight of 50–70 g by 6–8 months, although weight gain continues slowly after this age (Crofford and Davis 1965; Herberg et al. 1970). Hyperglycemia and glucose intolerance increase continuously with advancing age of the animals.

Renal disease in NZO mice is seen by 6 months of age. NZO mice have a high prevalence of autoimmune disorders.

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DIABETES OBESITY SYNDROME IN CBA/CA MICE

A spontaneous maturity onset diabetes obesity syndrome occurs in a small proportion (10–20%) of male CBA/Ca mice. Inbreeding can increase the incidence to 80%. It occurs at 12–16 weeks of age, and is characterized by hyperphagia, obesity, hyperglycemia, hypertriglyceridemia, hyperinsulinemia, and an impaired glucose tolerance. The mice are also resistant to exogenous insulin. Female mice remain normal except for a slight increase in serum insulin. The male obese diabetic mice have a normal life expectancy.

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FAT/FAT MICE

Fat mice carry an autosomal recessive mutation and display a rang of abnormalities, including progressive adult onset obesity, hyperinsulinemia and infertility (Coleman and Eicher 1990). The mutant allele of *fat* was identified and shown to be a missense (serin → proline) mutation in carboxypeptidase E which abolishes enzyme activity in a variety of neuroendocrine tissues (Naggert et al. 1995). Carboxypeptidase E is required for both sorting and proteolytic processing of a variety of prohormones including proinsulin and POMC (Cool et al. 1997). As carboxypeptidase E is expressed in the CNS, defective processing of a variety of hypothalamic neuropeptides – such as POMC and MCH – may trigger obesity in these animals (Rovere et al. 1996).

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TUBBY MICE

Tubby is an autosomal recessive mutation in mice (Coleman and Eicher 1990) which display a tripartite phenotype of blindness, deafness and maturity onset obesity. In response to weight gain, these mice gradually increase their food intake in proportion to body weight and increase plasma insulin levels thereby maintaining normoglycemia. The progressive retinal degeneration in *tubby* mice results from apoptotic loss of photoreceptor cells, with abnormal electroretinograms detected as early as 3 weeks of age (Heckenlively et al. 1955). The mouse obesity gene *tubby* has been identified and characterized (Noben-Trauth et al. 1996; Kleyn et al. 1996).

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L.2.0.4 Transgenic animals

PURPOSE AND RATIONALE

Transgenic animals will offer a new approach to study development of obesity and therapeutic possibilities. Lowell et al. (1993) used a transgenic toxigene approach to create transgenic mice with primary deficiency of brown adipose tissue.

The potential for inserting new genetic material into mammals has produced numerous transgenic mice with increased or decreased quantities of body fat (Bray and Bouchard 1997*)*.

Jensen et al. (1997) reported prevention of diet-induced obesity in transgenic mice overexpressing skeletal muscle lipoprotein lipase. The authors hypothesized that the potential to increase lipoprotein lipase activity in muscle by gene or drug delivery may prove an effective tool in preventing and/or treating obesity in humans.

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L.3 Assays of anti-obesity activity

L.3.1 Anorectic activity

L.3.1.1 Food consumption in rats

PURPOSE AND RATIONALE

Food intake is measured in acute experiments in normal or obese rats. Additionally, in semichronic experiments body weight gain is recorded.

PROCEDURE

Female Zucker rats weighing 250–350 g are maintained under standard conditions (temperature, light-dark cycle, ground rodent pellet chow, tap water). Measurements of food intake begin on day –2. The food is offered in special dishes to reduce spillage. Food intake and body weight are measured daily between 8:00 and 9:00 A.M. At this time any spilled food from the collecting paper under the cage is gathered, air-dried if necessary, and weighed. Individual food intakes in grams are recorded. The tests compounds are either administered with the food or injected intraperitoneally. Groups of 5–10 animals are used for control or treatment with various doses of the test compound or the standard. Mazindol, 3 m/kg i.p. or 10 mg/kg p.o. can serve as standard. Treatment is continued for 7 days.

EVALUATION

Average food intake and body weight is recorded for each day. Average values of test drugs are compared statistically for each day with the control group. Results after oral administration have to be confirmed by parenteral route in order to exclude errors due to palatability.

MODIFICATIONS OF THE METHOD

Hull and Maher, Maher and Hull (1990) used male Sprague-Dawley rats placed on a mush diet composed of equal parts of ground rodent chow and of 4% nutrient agar solution. The agar-based chow allows a more accurate measurement of food intake and has been shown to be sufficient for maintaining normal growth in rats. The rats were made hyperphagic by food deprivation for 4 h at the beginning of the dark cycle.

Mennini et al. (1991) and Anelli et al. (1992) studied the anorectic activity of various compounds in different species, such as mice, rats, and guinea pigs.

Bowden et al. (1988) used metabolism cages equipped with automated feeding monitors. Food was provided as 45 mg pellets which were singly delivered to a feeding trough. A photodetector sensed the removal of the pellet, and the number of pellets delivered over a specified time interval was recorded.

Samanin et al. (1979) described anorexia in rats induced by the central serotonin agonist m-chlorophenylpiperazine.

Dourish et al. (1985) investigated the effects of the serotonin agonist 8-OH-DPAT on food intake in nondeprived male rats. This effect was prevented by p-chlorophenylalanine (Dourish et al. 1986).

The anxiolytics gespirone, buspirone and ipsapirone increased free feeding in rats and did not inhibit feeding induced by 8-OH-DPAT (Gilbert and Dourish 1987).

Jackson et al. (1997) investigated the mechanisms underlying the hypophagic effects of the 5-HT and noradrenaline reuptake inhibitor, sibutramine, in the rat.

Simansky and Vaidya (1990) tested the anorectic action of a serotonin uptake inhibitor by measuring the volume of milk consumed by food-deprived rats.

Stevens and Edwards (1996) induced anorexia by subcutaneous injection of 5 mg/kg 5-hydroxytryptamine in Wistar rats habituated to a restricted feeding regime and tested the effects of a 5-HT_3 antagonist.

Rouru et al. (1992) investigated in genetically obese male Zucker rats the effect of subchronic metformin treatment on food intake, weight gain and plasma insulin and corticosterone levels and somatostatin concentrations in the pancreas.

Robert et al. (1989) found an enhanced food intake after intracerebroventricular administration of the tetrapeptide FMRF-amide (Phe-Met-Arg-Phe-NH_2) in obese "cafeteria" rats.

Cooper et al. (1990a,b) used non-deprived rats to study anorectic effects in a test of palatable food consumption and in nocturnal free-feeding.

Cooper et al. (1990c) tested not only food consumption but also the frequency of feeding bouts and duration of individual feeding episodes.

Eberle-Wang and Simansky (1992) studied the influence on the anorectic action of CCK and serotonin by measuring the uptake of sweetened mash mixture in rats.

Voigt et al. (1995) studied the involvement of the 5-HT_{1A} receptor in CCK induced satiety by recording food intake during a 2 h test meal in food deprived and in freely feeding rats.

Influence on postprandial satiety in rats was tested by Rosofsky and Geary (1989). Rats were given pelleted chow and water ad libitum. Near the middle of the bright phase of the light-dark cycle, pellets were removed, the animals treated and condensed milk presented 30 min later. Milk consumption was measured at 4-min intervals for 40 min.

Rats show a dramatic and reliable reduction of food intake if they are prefed a low-protein basal diet and then offered a diet that is imbalanced in any of the essential amino acids (Leung and Rogers 1969). This anorectic response has been used by Hammer et al. (1990) to test serotonin_3 receptor antagonists.

Tail pinch is an effective stimulant of eating in rats (Antelman and Szechtman 1975; Fray et al. 1982). Clark et al. (1992) reported that N-methyl-D-aspartate lesions of the lateral hypothalamus do not reduce amphetamine or fenfluramine anorexia but enhance the acquisition of eating in response to tail pinch in the rat.

Thurlby and Samanin (1981) studied the effect of anorectic drugs on food-rewarded runway behavior.

Ferrari et al. (1992) studied the effects on anorexia induced by ACTH and immobilization in rats in an X-maze with alternate open and covered arms, each baited with laboratory chow.

Cooper et al. (1993) studied dopamine D-1 receptor antagonists in rats with chronic gastric fistula which were trained to sham-feed a 10% sucrose solution in a 60 min test.

In wild rodents, **hoarding of food** covers the long term alimentary need. In the laboratory, hoarding behavior does not occur in ad libitum fed rats. On the contrary, rats whose energy balance is threatened by previous food restriction hoard as soon as experimental conditions allow to do so. When such a rat gets free access to a food stock (placed outside its usual territory), it carries food into its shelter and accumulates an amount proportionate to its body weight. Fantino et al. (1980, 1986, 1988), Nishida et al. (1990) used the reduction of the amount of food hoarded during a period of 3 h as parameter for anorectic activity of drugs.

Caccia et al. (1993) studied the anorectic effect of D-fenfluramine in the **marmoset** (*Callithrix jacchus*).

Knoll (1979, 1984) described satietin, a anorectic substance which has been isolated from serum of humans and several animal species. Satietin has been reported to be a 50 000–70 000 dalton MW glycoprotein, containing 14–15% amino acids and 70–75% carbohy-drates, surviving digestion with proteases and boiling. Purification of bovine serum satietin was reported by Nagy (1994). These factors suppressed feeding in rats after peripheral and after intracerebroventricular administration.

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L.3.2 Metabolic activity

L.3.2.1 GDP-binding in brown adipose tissue

PURPOSE AND RATIONALE

Brown adipose tissue is the major site for non-shivering thermogenesis in rodents (Ricquier and Mory 1984; Foster 1986). Drugs activating brown adipose tissue thermogenesis via β-adrenoceptors cause uncoupling of oxidative phosphorylation from electron transport (Arch et al. 1984; Nicholls et al. 1986). The binding of the nucleotide guanosine diphosphate (GDP) to brown adipose tissue membrane protein, the uncoupling protein or thermogenin (Ricquier and Bouillaud 1986), is an established indicator of the thermogenetic activity of brown adipose tissue (Milner et al. 1988).

PROCEDURE

Obese male fatty Zucker rats at the age of 13 weeks weighing about 450 g receive various doses of the test compound in the drinking water or tap water as control for 21 days. Food intake is measured every day and body weight every other day. At the end of the treatment, the rats are sacrificed by decapitation and interscapular brown adipose tissue is quickly dissected from surrounding tissue.

According to the method published by Nicholls (1976) brown adipose tissue is minced, diluted with 250 mM sucrose and homogenized with a Potter S homogenizer (B. Braun Melsungen, Germany). The homogenate is centrifuged for 10 min at 8 500 *g*. The pellet is diluted with 250 mM sucrose and centrifuged at 700 *g* for 10 min. The supernatant is collected and centrifuged at 8 500 *g* for 10 min. Bovine serum albumin at 0.2% is added to wash the suspension of the pellet, which now consists of mitochondria. After centrifugation (8 500 *g*) the resulting pellet is suspended in albumin-free sucrose buffer. The binding of [3H]-GDP (DuPont NEN, Boston MA) to mitochondria of single rats is determined by incubating mitochondria in a basic medium containing 100 mM sucrose, 20 mM TES (*N*-tris-[hydroxymethyl]-methyl-2-aminoethane-sulphonic acid), 1 mM EDTA, 10 mM choline chloride, 2 μM rotenone, [^{14}C]sucrose (0.125 μCi ml^{-1}) and 10 μM [^{3}H]-GDP (0.53 Ci mmol^{-1}) at room temperature. Non-specific binding is assessed in the presence of unlabelled GDP (1 mM). After 10 min of incubation, the reaction is terminated by filtration the mixture through Thomas A/E glass fibre filters by using a Brandel cell harvester. The radioactivity is measured with the Opti Phase 'High Safe' II scintillation cocktail and a scintillation counter enabling the samples to be counted for both ^{3}H and ^{14}C. The protein content of the mitochondrial suspensions is assayed according to the method of Peterson (1977).

EVALUATION

GDP binding is assessed from ^{3}H radioactivity with a correction for trapped medium using [^{14}C]-sucrose. Two way analysis of variance (ANOVA) is performed.

MODIFICATIONS OF THE METHOD

Glucose is a minor substrate for isolated brown adipocytes, fuelling thermogenesis by a maximum of 16% (Isler et al. 1987).

The mechanism of anti-obesity action of a dihydropyridine calcium antagonist was studied by Yoshida et al. (1994b). Obesity was induced by subcutaneous injection of 2 g/kg monosodium-L-glutamate immediately after birth for 5 consecutive days in ICR female mice. Binding of GDP and cytochrome c oxidase activ-ity in brown adipose tissue mitochondria were significantly increased after 4 weeks drug treatment incorporated in the diet.

Yoshida et al. (1984, 1985) found a reduced norepinephrine turnover in brown adipose tissue of mice with monosodium glutamate-induced obesity.

Kajita et al. (1994), Takahashi et al. (1994) determined regional blood flow in brown adipose tissue by the microsphere method in anesthetized rats.

Yoshida et al. (1996) determined the effect of mazindol on noradrenaline turnover in brown adipose tissue of yellow KK mice and C57B1 control mice.

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L.3.2.2 Uncoupling protein and GLUT4 in brown adipose tissue

PURPOSE AND RATIONALE

Uncoupling proteins (UCPs) are a family of inner mitochondrial membrane transporters which dissipate the

proton gradient, releasing stored energy as heat. UCP1 is expressed exclusively in brown adipocytes, UCP2 is expressed widely, while UCP3 is found in skeletal muscle and brown adipose tissue (Vidal-Puig et al. 1997; Fleury et al. 1997; Masaki et al. 1997; Matsuda et al. 1997; Boss et al. 1997; Gong et al. 1997; Larkin et al. 1997). They are upregulated by thyroid hormones (Larkin 1997; Masaki 1997; Branco et al. 1999; Lanni et al. 1999). Mao et al. (1999) identified and characterized a novel member of the human uncoupling protein family, termed uncoupling protein-4 (UCP4).

In addition to the binding of the nucleotide guanosine diphosphate (GDP) to brown adipose tissue membrane protein, the uncoupling protein itself, and the glucose transporter 4 (GLUT4) were determined by RNA (Northern blot) analysis and by protein (Western blot) analysis as indicators of the thermogenetic activity of brown adipose tissue.

PROCEDURE

Male fatty (OLETF fatty) rats at the age of 10 weeks are given subcutaneous injection of test compound or solvent once daily. After 14 weeks treatment, the rats are sacrificed and brown and white adipose tissue samples rapidly removed.

Northern blot analysis

Total RNA is extracted from 0.1–1 g of tissue using TRIzol (Gibco) and the concentration determined from the absorbance at 260 nm. Total RNA (20 mg) is separated on a 1.5% agarose/formaldehyde gel, and transferred to and fixed on a nylon membrane. A 488 bp uncoupling protein cDNA probe corresponding to the coding region of rat uncoupling protein is prepared by digesting the whole uncoupling protein cDNA with BamHI. The uncoupling protein probe and GLUT4 cDNA are labeled with α-[^{32}P]dCTP (deoxy-cytidine-triphosphate). The blots are hybridized to the labeled probes at 42 °C for 20 h in the presence of 500 µg/ml salmon sperm DNA, and exposed to an X-ray film for autoradiography and an imaging plate of BAS1 000 (Fuji Film) for quantitative analysis.

Western blot analysis

Each tissue is homogenized in 5–10 volumes of a solution containing 10 mmol/l Tris-HCl and 1 mmol/l EDTA (pH 7.4) for 30 s with a Polytron. After centrifugation at 1 500 *g* for 5 min, the fat cake is discarded, and the infranatant (fat-free extract) is used for protein determination according to Lowry et al. (1951) and cytochrome C oxidase activity (Yonetani and Ray 1965). Uncoupling protein and GLUT4 protein in the fat-free extract is measured by Western blot analysis. The fat-free extracts (10 µg protein of brown adipose tissue, 20 µg of white adipose tissue) are solubilized, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose filter. After blocking with 5% non-fat dry milk, the filter is incubated with rabbit antiserum against rat uncoupling protein or GLUT4. The rabbit antisera against rat uncoupling protein and GLUT4 are prepared by immunizing purified rat uncoupling protein (Lin and Klingenberg 1980) and a 12-amino acid peptide corresponding to GLUT4 (residues 498–509, TELEYLGPDEND) (Shimizu et al. 1993) respectively, coupled with keyhole limpet hemocyanin. Then the filter is incubated with [^{125}I]protein A (ICN). The dry blot is exposed to an X-ray film for autoradiography and an imaging plate of BAS1 000 (Fuji Film) for quantitative analysis.

EVALUATION

Data are expressed as means ±SEM and are analyzed by two-way ANOVA followed by Bonferroni *t*-test.

MODIFICATIONS OF THE METHOD

A radioimmunoassay to measure uncoupling protein was used by Milner et al. (1988).

Scarpace et al. (2000) performed unilateral surgical denervation of the interscapular BAT in rats under pentobarbital anesthesia according to Bartness et al. (1986). A transverse incision was made just anterior to the BAT, separating the BAT from the muscles of the scapulae. The BAT was raised to expose the five intercostal nerve bundles entering each pad. On one side, a section of each nerve bundle was removed with scissors. Denervation can be verified by assessing norepinephrine levels in the innervated compared with the denervated BAT pads (Scarpace and Matheny 1996).

Puigserver et al. (1996) studied the effects of retinoic acid isomers on the appearance of uncoupling protein in primary cultures of brown adipocytes, in the brown adipocyte cell line HIB 1B and directly in intact mice.

Shimabukuro et al. (1997) reported the induction of uncoupling protein-2 mRNA in the pancreatic islets of Zucker diabetic fatty rats by troglitazone.

Foellmi-Adams et al. (1996) found a synergy between norepinephrine and pioglitazone in induction of uncoupling protein in mice.

Kotz et al. (2000) determined uncoupling protein 1 (UCP1) in brown adipose tissue, UCP2 in white adipose tissue and UCP3 in muscle of male Sprague Dawley rats after injection of neuropeptide Y into the hypothalamic paraventricular nucleus.

Great efforts have been devoted to study the influence of **β_3-adrenergic agonists** on formation of uncoupling protein.

Umekawa et al. (1997) found an induction of uncoupling protein and activation of GLUT4 in white fat

of Otsuka Long-Evans Tokushima fatty rats after treatment with a specific β_3-adrenoceptor agonist.

Ghorbani and Himms-Hagen (1997) found appearance of abundant densely-stained brown adipocytes expressing uncoupling protein in white adipose tissue during reversal of obesity and diabetes in Zucker fa/fa rats induced by the β_3-adrenoceptor agonist CL316,243.

Nagase et al. (1996) found in yellow obese KK mice after treatment with a β_3-adrenergic agonist a significant reduction of body weight, associated with a marked decrease of white fat pad weight and hypertrophy of the interscapular brown adipose tissue with a sixfold increase in mitochondrial uncoupling protein content.

Liu et al. (1995, 1996), Stock (1997) reported an increase of glucose utilization in rat brown adipose tissue after treatment with a β_3-adrenoreceptor agonist or with sibutramine, a serotonin and noradrenaline reuptake inhibitor.

Savontaus et al. (1998) reported an increase of UCP3 and UCP1 in brown adipose tissue of obese fa/fa Zucker rats after chronic administration of a β_3-adrenoreceptor agonist.

The effects of β_3-adrenoceptor agonists on uncoupling protein-1 and leptin in culture-differentiated rat brown fat cells are antagonized by a β_3-adrenoreceptor antagonist (Tonello et al. 1998).

Berraondo et al. (2000) found an up-regulation of muscle UCP2 gene expression by a β_3-adrenoreceptor agonist in obese rodents, but down-regulation in lean animals.

Paulik and Lennard (1997) found an increased expression of uncoupling protein in C3H10T1/2 cells, a pluripotent stem-cell line, after addition of **thiazolidinediones**.

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L.3.2.3 Resting metabolic rate

PURPOSE AND RATIONALE

Resting metabolic rate can be influenced by various drugs both in normal and obese animals.

PROCEDURE

Female yellow KK mice and female C57B1 mice at the age of 12 weeks are housed under controlled temperature and artificial light/dark conditions and are fed with commercial powdered chow and tap water ad libitum. Groups of each strain are treated with doses of test drug or solvent by daily intramuscular injections for 2 weeks. Daily food intake and body weight is measured.

Resting metabolic rate is estimated by means of a closed-circuit metabolic system (Molnar et al. 1986). The system consists of a chamber, circulating pump, desiccant and CO_2-absorbent canisters, solenoid valve, regulated gas source, and modified liquid crystal display calculator. The internal volume of a chamber for a single rat should be 4.5 liters. Food cups are placed on the door for convenient access when refilling. Water is supplied in a tray filled intermittently by means of a stopcock in the chamber wall. A shallow stainless steel funnel-shaped tray for urine collection is located in the bottom of the chamber.

Animals are placed in the chamber and the door is sealed airtight. Chamber air circulates by means of a peristaltic pump at a rate of 2.3 l/min through an external loop containing canisters of desiccant and CO_2-absorbent. In this manner, water vapor pressure remains constant and the respiration by-product, CO_2, is removed. Consumption of O_2 produces a pressure drop in the sealed system which actuates the pressure sensor for O_2 replacement.

The pressure sensor has two input ports, low and high, producing a simple contact closure whenever the high pressure exceeds the low by ~1 Torr. The low port is connected to the interior of the metabolic chamber by a length of tubing attached to a threaded connector, whereas the high port is open to the atmosphere. Thus the contact closes whenever the pressure within the chamber falls to more than 1 Torr below atmospheric pressure.

The contact closure is routed to the control unit where a precise pulse of fixed duration is triggered. This pulse activates a solid-state relay through an emitter-follower transistor circuit. The relay output is in series with a solenoid valve and line voltage, so that contact closure results in opening of the valve for a fixed duration. Since the input port of the valve is at a constant pressure, a predictable, highly repeatable volume of gas flows into the chamber. This raises the chamber pressure and opens the pressure sensor contacts. The entire cycle repeats after sufficient metabolism to again decrease the chamber pressure and close the sensor contacts.

After a stable baseline has been achieved after 30 min, drugs are administered and resting metabolic rate is measured for one hour at an ambient temperature of 22 °C.

EVALUATION

Data are presented as means ±SEM. Statistical analysis is performed by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Poon and Cameron (1978) measured oxygen consumption utilizing a standard Machlett manometer connected to a desiccator that accommodated a single mouse. The desiccator was placed in a 20 °C water bath to ensure constant temperature throughout and CO_2 was absorbed by means of soda lime UPS. After a 5-min equilibration period, measurements of oxygen consumption were obtained over a 5-min period. The procedure was repeated twice within 1 h for each mouse.

In order to study differential antagonism to amphetamine-induced oxygen consumption and agitation by psychoactive drugs Niemegeers and Janssen (1979) placed groups of 3 rats with a total weight of 235 ±5 g consisting of 2 parts, a bottom and a cover, both made up from clear glass. The cover, containing a thermometer, was used to seal the chamber airtight. Inside the chamber a perforate plate was placed as animal platform. Containers filled with calcium oxide were placed below and above the animal holder. The chamber was flushed with oxygen for 3 min at an overpressure of 50 units as measured by a manometer connected to one outlet of the chamber. Test drugs were administered subcutaneously 60 min before the animals were placed in the test chambers and 45 min before 2.5 mg/kg amphetamine were injected. Oxygen consumption was measured as decrease of manometer pressure over one hour.

Rothwell (1989) measured central activation of thermogesesis by prostaglandins by resting oxygen consumption in individual closed-circuit calorimeters (Stock 1975) and by registration of colonic temperature in conscious rats.

Jensen et al. (2001) described a self-correcting indirect calorimeter system for the measurement of energy balance in small animals.

Himms-Hagen et al. (1994) tested the effect of a thermogenic β_3-agonist on energy balance in rats. Twenty-four hours energy expenditure, resting metabolic rate, and thermic effect of food were measured using open-circuit indirect calorimetry. The rat was placed in a respiration chamber ($44 \times 22 \times 18$ cm), airflow was measured with a Brooks thermal mass flowmeter (Brooks Instrument Division, Emerson Electric, Hatfield, PA), and the flow of outside air through the chamber was controlled at a variable rate by two adjustable peristaltic pumps that maintained CO_2 concentration at <1%. Temperature was maintained at 23 ±1.0 °C. Humidity was measured by an electronic psychrometer, barometric pressure was recorded, and feeding activity was monitored by an infrared detector. The rat was placed into the chamber at 09:30 and removed at 08:30 at the following morning. Food was available to the rat only between 16:30 and 07:30. Total energy expenditure was measured for 23. Resting metabolic rate was determined from the lowest energy expenditure at rest between 11:00 and 16:00. Thermic effect of food was determined from the difference between resting metabolic rate and the lowest energy expenditure at rest between 24:00 and 07:00. For the measurement of minimum metabolic rate, the rat was anesthetized (pentobarbital sodium, 60 mg/kg) and then placed in a small chamber filled with warm circulating water in which it was submerged except for the head. Core temperature was measured with a digital thermometer immediately after induction of anesthesia, and this temperature was maintained (±0.1 °C) by adjusting the temperature of the circulating water bath. The chamber was sealed and minimum metabolic rate determined with the same equipment as was used for the 24-h energy expenditure. Measurements of minimum metabolic rate were made for 5–15 min between 08:30 and 09:30 after a stabilization period of 5–10 min.

Ghorbani et al. (1997) tested the effect of a β_3-adrenoreceptor agonist on resting metabolic rate in rats by placing the animal in a water-jacketed chamber at 28 °C, volume 1 liter, through which air, also at 28 °C, was drawn at a rate of 1 l/min. Oxygen entering and leaving the chamber was measured with an oxygen analyzer (Beckman Industrial Oxygen Analyzer, model 755).

Paulik et al. (1998) described a robust technique to measure thermogenesis of **yeast cells** cultured in microtiter plates using infrared thermography. Thermogenesis increased after exposing yeast to uncoupling protein-2, or troglitazone or β_3-adrenoreceptor agonists.

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L.3.2.4 β_3-adrenoceptor

PURPOSE AND RATIONALE

β_3-adrenoreceptor agonists produce weight loss in obese rodents (Yoshida et al. 1994; Weyer et al. 1999) which is almost entirely accounted for a reduction in body fat. As this effect is observed without a decrease in food intake, it is thought to be due to increased thermogenesis in brown adipose tissue, increased lipolysis in white adipose tissue, and suppression of leptin gene expression and serum leptin levels (Arch and Wilson 1996; Kumar et al. 1999).

The β_3-adrenoreceptor has been cloned and characterized in animals (Granneman et al. 1991; Nahmias et al. 1991; Evans et al. 1996) as well as from man (Emorine et al. 1989; Strosberg 1997). However, the physiological function of the human β_3-adrenoreceptor, the significance of animal data with β_3-adrenoreceptor agonists, and even the presence of a putative fourth β-adrenoreceptor in human adipose tissue are still a matter of debate (Emorine et al. 1994; Galitzky et al. 1998; Kaumann et al. 1998; Sarsero et al. 1998; Strosberg et al. 1998; Weyer et al. 1999).

The development of a Chinese hamster ovary cell transfection system, using the human β_3-adrenoreceptor gene resulted in potential new selective β_3-agonists being identified (Carruba et al. 1998). Human β_1-, β_2- and β_3-adrenoreceptors expressed in Chinese hamster ovary (CHO) cells were used to evaluate potential β_3-adrenoreceptor agonists by He et al. (2000).

PROCEDURE

cAMP response element-luciferase receptor gene assay

CHO cells stably expressing human β_1-, β_2- or β_3-adrenoreceptor populations are transfected with cAMP response element-luciferase plasmids using electroporation with a single 70-ms, 150-V pulse (Vansal and Fellner 1999). The transfected cells are seeded at a density of 40 000/well in 96-well microtiter plates and allowed to grow for 20 h. After 20 h, the cells are treated with varying drug concentrations (10^{-11} to 10^{-4} M) for 4 h. Following drug exposure, the cells are lysed and luciferase activity is measured using the LucLite assay kit (Packard). Changes in light production are measured by a Topcount luminometer (Packard).

EVALUATION

Data are analyzed in duplicate at each concentration and expressed as percent luciferase response relative to the maximum response to (–)-isoproterenol (10^{-6} M). Results are expressed as the mean ±SEM.

MODIFICATIONS OF THE METHOD

Gettys et al. (1996) reported that the β_3-adrenergic receptor inhibits insulin-stimulated leptin secretion from isolated rat white adipocytes.

Using reverse transcription-polymerase chain reaction, Evans et al. (1998) compared levels of β_3-adrenoreceptor mRNA in white adipose tissue, brown adipose tissue, ileum and colon from genetically obese (*ob/ob*) and lean (+/+) C57BL/6J mice.

Bioassays

Tomiyama et al. (1998) studied β-adrenoreceptor subtypes mediating ureteral relaxation in rats, rabbits and dogs. β_3-adrenoreceptor agonists were more effective in relaxing canine ureter than β_1- and β_2-adrenoreceptor agonists.

Koike et al. (1997) reported that the relaxant responses in the guinea pig taenia caecum are mediated by both the β_2- and β_3-adrenoreceptors.

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L.4 Assays of obesity regulating peptide hormones

L.4.0.1 Hormonal regulation of food intake

Food intake and fat deposition are regulated by neurotransmitters peptides, most of the located in the brain, particularly in the hypothalamus (Elmquist et al. 1999; Kalra et al. 1999) and in the gut. This includes peptides that are **orexigenic** (appetite-stimulating) signals and **anorectic** peptides.

Neuropeptide Y (NPY), orexins A and B, galanin, melanin concentrating hormone (MCH), and **agouti-related peptide (AgRP)** all act to stimulate feeding, while **alpha-melanocyte stimulating hormone (αMSH)**, (see N.10.1), **corticotropin releasing hormone (CRH)**, (see N.9.4), **cholecystokinin (CCK)**, (see J.7.0.10), **cocaine and amphetamine regulated transcript (CART), neurotensin, glucagon-like peptide 1 (GLP_1)**, (see K.4.0.3), **calcitonin gene related peptide (CGRP)**, (see N.10.3), **bombesin** (see J.3.1.8) and **ciliary neurotropic factor** (Xu et al. 1998) have anorectic actions (Tritos et al. 1999).

Mutations reducing the functional activity of leptin, the leptin receptor, α-MSH, and the melanocortin-4 receptors lead to obesity in animals. *Mc4r*-deficient ($Mc4r^{-/-}$) mice do not respond to the anorectic actions of MTII, an MSH-like antagonist, suggesting that α-MSH inhibits feeding primarily by activating Mc4r (Marsh et al. 1999).

Leptin, (see L.4.1) is a 167 amino acid protein that is synthesized and secreted primarily by white adipose tissue, circulates in the blood and acts on receptors in the hypothalamus to decrease food intake and increase energy expenditure (Friedman and Halaas 1998; Trayhurn et al. 1999). Leptin and its derivatives are candidates for treatment of obesity, however, clinical studies (Considine et al. (1996) showed that most of obese humans have higher plasma levels of leptin than non-obese individuals, suggesting that obesity is associated with leptin resistance rather than leptin deficiency.

Neuropeptide Y (NPY) (see L.4.2) is a 36 amino acid peptide that is widely distributed throughout both the central and peripheral nervous systems and which plays a key role in the control of body weight. Central administration of NPY increases food intake (Stanley et al. 1992), while a reduction in endogenous neuropeptide Y leads to a decrease of food intake (Lambert et al. 1993). NPY antagonists are candidates for anti-obesity drugs.

Orexin-A and orexin-B (see L.4.3) are 33- and 28-residue peptides, also called hypocrites, which were originally isolated from rat hypothalamus (Sakurai et al. 1998). These peptides are located predominantly in the hypothalamus and locus coeruleus but are also found elsewhere in the brain. The orexins have a broad range of physiological functions, including the control of feeding and energy metabolism. Food consumption is dose-dependently increased after intracerebroventricular infusion of orexin A and orexin B to rats (Jain et al. 2000). Orexin antagonists are potential anti-obesity drugs (Parker 1999).

Galanin (see L.4.4) is 29 amino acid C-terminally amidated peptide (30 amino acids in humans) which is localized mainly in the mammalian CNS, but also in other organs. Central administration of galanin increases and administration of galanin receptor antagonists (Crawley et al. 1990; Leibowitz and Kim 1992) decreases food intake. These data suggest the use of galanin receptor antagonists as anti-obesity agents.

Agouti-related protein affects pigmentation when its expression is limited to the skin, but ubiquitous expression causes obesity (Ollmann et al. 1997). The hypothalamic expression of agouti related protein is regulated by leptin, and overexpression of agouti related protein results in obesity and diabetes (Rosenfeld et al. 1998). Recombinant Agouti-related protein is a potent, selective antagonist at MC3R and MC4R, melanocortin receptor subtypes implicated in weight regulation (Fong et al. 1997; Shutter et al. 1997; Tota et al. 1999). Ubiquitous expression of human Agouti-related protein complementary DNA in transgenic mice caused obesity without altering pigmentation. Agouti-related protein is a neuropeptide implicated in the normal control of body weigh downstream of leptin signaling. Ollmann et al. (1998) used a sensitive bioassay based on *Xenopus* melanophores to characterize pharmacological properties of recombinant Agouti protein.

Melanin concentrating hormone (MCH) is a cyclic 19 amino acid neuropeptide that was originally found to regulate pigmentation in fish. It plays a role in the central feeding behavior increasing food consumption (Qu et al. 1996; Rossi et al. 1999). Mice carrying a targeted deletion of the MCH gene are hypophagic and lean (Shimada et al. 1998). Among other factors, MCH may be a target of leptin signaling in the hypothalamus (Sahu 1998; Huang et al. 1999). Melanin-concentrating hormone is a functional melanocortin antagonist in the hypothalamus (Ludwig et al. 1998). MCH has been identified as the natural ligand for the orphan somatostatin-like receptor 1 (SLC-1) (Bacher et al. 1999; Chambers et al. 1999; Shimomura et al. 1999) which has been characterized by Saito et al. (1999). High binding capacity for MCH was found in human keratinocytes (Burgaud et al. (1997). Radioligands for the mammalian MCH receptor were described by Hintermann et al. (1999).

Cocaine- and amphetamine-regulated transcript (CART), a brain located peptide, has potent appetite suppressing activity and is closely associated with the actions of leptin and neuropeptide Y (Lambert et al. 1998; Kuhar and Dall-Vechia 1999). When injected intracerebroventricularly into rats, recombinant CART peptide inhibits both normal and starvation-induced feeding, and completely blocks the feeding response induced by neuropeptide Y (Kristensen et al. 1998). In the rat the CART gene encodes a peptide of either 129 or 116 amino acids whereas only the short form exists in humans (Thim et al. 1998a,b). A role of CART peptides in substance abuse and addiction is suggested by psychomotor-stimulant regulation of CART transcription in the striatum, as well as its localization within neural circuits that mediate reward and reinforcing behaviors (Couceyro and Lambert 1999). CART has been found not only in the hypothalamus and other brain areas (Broberger 1999; Koylu et al. 1998, 1999) but also in other organs, such as the pancreas (Jensen et al. 1999), in the rat sympatho-adrenal axis (Dun et al. 2000), or in the vagal afferent neurons sensitive to cholecystokinin (Broberger et al. 1999). CART can cross the blood-brain barrier (Kastin and Akerström 1999). CART receptor agonists are potential anti-obesity drugs (Couceyro and Lambert 1999).

Steppan et al. (2001), Berger (2001) showed that adipocytes secrete a signaling molecule with 114 amino acids in length which is called **resistin** (for resistance to insulin) and which links obesity to diabetes. Circulating resistin levels in mice are increased in diet-induced and genetic forms of obesity and are decreased by thiazolidindiones (see K.5.0.6). Administration of anti-resistin antibody improves blood sugar and insulin action in mice with diet-induced obesity. Treatment of normal mice with recombinant resistin impairs glucose tolerance and insulin action. Insulin-stimulated glucose uptake by adipocytes ins enhanced by neutralization of resistin and is reduced by resistin treatment. Resistin with slightly modified amino acid sequence has also been found in humans.

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L.4.1 Leptin

L.4.1.1 General considerations on the obese gene product leptin

The obese gene product leptin is a cytokine-like nonglycosylated peptide of 146 amino acids that is secreted from adipose tissue. It is an important circulating hormone for the regulation of body weight (Arch and Beeley 1996; Hamann and Matthaei 1996; Friedman and Halaas 1998; Trayhurn 1999). MacDougald et al. (1995) described the expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes.

The mouse obese gene and its human homologue which show approximately 85% amino acid homology have been cloned by Zhang et al. (1994). Leptin binding was first reported in rat hypothalamic membranes (Stephens et al. (1995).

A **leptin receptor** was subsequently identified by expression cloning from a mouse chorioid plexus cDNA library (Tartaglia et al. 1995). Several transcripts of the leptin receptor as a result of alternative splicing which have different length of cytoplasmatic region have been identified in mice and rats (Guan et al. 1997; Igel et al. 1997; Murakami et al. 1997) as well as leptin receptors in man (Maffei et al. 1995, 1996; Liu et al. 1997; Strosberg and Issad 1999). There are at least five different isoforms of the leptin receptor in mice. Mutations in the leptin receptor lead to massive obesity in *db* mice and *fa* rats (Friedman and Halaas 1998).

Administration of recombinant human leptin to mice increases thermogenesis and lipid oxidation in brown fat coupled with increased lipolysis and decreased fat synthesis in white and brown fat, which lead to a rapid reduction in the body weight (Sarmiento et al. 1997).

Leptin increases uncoupling protein expression in brown adipose tissue and oxygen consumption in mice (Scarpace et al. 1997).

Intracerebroventricular injection of recombinant murine leptin produces a dose-dependent reduction of laboratory diet in normal rats. This effect is attenuated in rats with diet-induced obesity (Widdowson et al. 1997).

The role of leptin in human obesity is controversial. Rare forms of human obesity have been identified where mutations in leptin or its receptor play a major role in the development of the disease. Leptin may be a signal for the onset of puberty in humans (Strosberg and Issad 1999). Leptin activates leptin-receptor expressing neurons in the arcuate region of the hypothalamus. Projections from these neurones stimulate melanocortin MC_4 receptors and neuropeptide Y-containing neurones to activate the sympathetic nervous system which controls lipolysis in white adipose tissue. The firing of gonadotrophin-releasing hormone-containing neurons and secretion of gonadotrophin-releasing hormone to the pituitary is elicited by leptin-mediated activation of leptin-receptor-expressing neurons and by other factors that control the reproductive axis (Chebab 2000).

Grasso et al. (1997, 1999) found inhibitory effects of leptin-related synthetic peptides on food intake and body weight gain after intraperitoneal administration in female C57BL/6J*ob/ob* mice which may have potential application to the treatment of human obesity.

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L.4.1.2 Determination of leptin mRNA level in adipose tissue

PURPOSE AND RATIONALE

Leptin mRNA levels in adipose tissue can be determined by Northern blot analysis (Friedrich et al. 1995; Harris et al. 1996; Zachwieja et al. 1997; Kochan et al. 1999).

PROCEDURE

Male Wistar rats weighing approximately 230 g treated for 14 days with test drug are sacrificed by decapitation. Liver, epididymal white adipose tissue and intracapsular brown adipose tissue are rapidly removed and frozen in liquid nitrogen. Total RNA is extracted from frozen tissues by a guanidinium thiocyanate-phenol/chloroform method (Chomczynski and Sacchi 1987). The RNA (10 µg per lane) is fractionated by horizontal gel electrophoresis. The RNA is transferred to a positively charged nylon membrane and fixed with UV-light. Pre-hybridization is performed at 42 °C for 45 min in pre-hybridization solution containing 7% sodium dodecyl sulfate, 50% formamide, 5 × saline-sodium citrate buffer, 2% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 50 mM sodium phosphate (pH 7.0) and 0.1% N-laurylsarcosine. Hybridization is performed at 42 °C overnight in pre-hybridization solution containing oligonucleotide probe (25 ng/ml) specific to leptin, malic enzyme or 185 RNA. The following post-hybridization washes are performed: twice for 5 min in 2 × saline-sodium citrate buffer/0.1% sodium dodecyl sulfate (at room temperature): twice for 15 min in 0.1 × saline-sodium citrate buffer/0.1% sodium dodecyl sulfate (at 48 °C). The membranes are then rinsed briefly with washing buffer containing 0.1 M maleic

acid (pH 7.5), 0.15 M NaCl and 0.3% Tween 20. They are blocked by incubation for 30 min at room temperature with blocking buffer (1% blocking agent, 0.1 M maleic acid (pH 7.5), 0.15 M NaCl) and incubated (at the same conditions as described above) with blocking buffer containing a polyclonal antibody against digoxigenin conjugated to alkaline phosphatase. After washing twice for 15 min with washing buffer, the membranes are rinsed for 5 min with detection buffer (0.1 M Tris-Cl, pH 9.5, 0.1 M NaCl) and immersed for 5 min in CDP-Star solution (Boehringer Mannheim, Mannheim, Germany). Membranes are exposed to Kodak XAR film for 15 min to 1 h (Karbowska et al. 1999).

EVALUATION

Signals are scanned and quantified using the NIH-Image software. The level of mRNA for leptin and malic enzyme as well as for 185 RNA is estimated using PeakFit software (Jandel Scientific). The values for leptin and malic enzyme mRNA are normalized to the corresponding amount of 185 RNA. Results expressed in arbitrary units are presented as means ±SEM of samples of 10 rats. The statistical difference between groups is assessed by one-way analysis of variance (ANOVA) followed by Student's *t*-test or by Mann-Whitney test.

MODIFICATIONS OF THE METHOD

Richards et al. (2000) described quantitative analysis of leptin mRNA using competitive reverse transcription polymerase chain reaction and capillary electrophoresis with laser-induced fluorescence detection.

Shintani et al. (2000) determined leptin mRNA levels in primary cultured rat adipocytes.

Li et al. (1999) used an anti-leptin polyclonal antiserum to detect leptin-immunoreactivity in the central nervous system in rats.

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L.4.1.3 Determination of plasma leptin

PURPOSE AND RATIONALE

Plasma leptin levels are determined by radioimmunoassay using a rat-specific antibody (Ciba-Geigy, Basel, Switzerland) (Zachwieja et al. 1997) or with a double-antibody radioimmunoassay kit based on a mouse standard (Linco Research, St. Louis, MO) (Surwit et al. 1997; Van Heek et al. 1997). A radioimmunoassay for leptin in human plasma was described McGregor et al. (1996).

PROCEDURE

The peptide VPIQKVQDDTKTLIKTIVT representing the first twenty amino acids of the predicted sequence of mature leptin protein (leptin 1–20) was synthesized with 9-fluorenyl methyl oxycarbonyl-(Fmoc)-protected L-amino acids on a applied Biosystems peptide synthesizer and purified by HPLC on a Dynamax column developed with a 0.1% trifluoroacetic acid (TFA)/H_2O/acetonitrile (8% to 40%) gradient. The peptide is conjugated to thyroglobulin using carbodiimide as coupling reagent. Rabbits are immunized by intradermal injections and boosted at 4 weeks intervals.

A peptide identical to the one used for antibody generation except for a tyrosine residue added to the C-terminal end is labelled with ^{125}I using the oxidizing agent Iodogen (Pierce, USA) and purified on HPLC. For radioimmunoassay, the buffer consists of Tris-HCl 1 M, pH 7.4; 0.1% gelatin; 0.1% Triton X-100, and 0.01% NaN_3. Unlabelled ob peptide is used for standards. 100 µl of standard or unknown, 200 µl buffer, 100 µl antibody at a final concentration of 1 : 80 000 and 100 µl ^{125}I-leptin peptide (approximately 5 000 cpm) are added to polystyrene tubes and incubated for 48 h at 4 °C. Antibody-bound and unbound peptide are sepa-

rated by addition of goat anti-rabbit immunoglobulin antibody followed by centrifugation. The precipitates are counted on a computer-linked gamma-counter and the leptin concentrations of the samples are obtained using the "RIA-Calc" program.

EVALUATION

Results are expressed as means ±SD, and statistical comparisons are made with the use of Student's *t*-test. Linear regression analysis is performed with the method of least squares.

MODIFICATIONS OF THE METHOD

Maffei et al. (1995) used an immunoprecipitation assay to measure plasma leptin levels. A quantitative increase of the signal intensity was seen on Western blots when increasing amounts of recombinant protein were added to *ob* mouse serum, which does not have leptin. Densitometry was used to compare the signal intensity of the native protein in mouse plasma to the standard. The level of leptin-like immunoreactivity was quantified as nanograms per ml.

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L.4.2 Neuropeptide Y

L.4.2.1 General considerations on neuropeptide Y and related peptides

Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) are endogenous 36-amino acid peptides belonging to the same family, which is characterized by a tertiary structure termed 'PP-fold' (Larhammar 1996). They have different tissue distributions (Sundler et al. 1993) and act in different physiological systems, mostly neuronal for NPY and endocrine for PYY and PP (Wettstein et al. 1995; Playford and Cox 1996; Goumain et al. 1998).

Neuropeptide Y plays a key role in the control of body weight (Leibowitz 1994). Central administration of neuropeptide Y increases food intake and decreases thermogenesis in satiated animals (Billington et al. (1991, Stanley et al. 1992), while a reduction in endogenous neuropeptide Y leads to a decrease of food intake (Lambert et al. 1993). Hypothalamic neuropeptide Y and mRNA levels are increased after fasting and in genetically obese mice (Stephens et al. 1995). Neuropeptide Y is required for the maintenance of the obese phenotype of the leptin-deficient *ob/ob* mice (Erickson et al. 1996). Conversely, leptin appears to decrease food intake and body weight in part by decreasing neuropeptide Y synthesis and release (Stephens et al. 1995). These data suggest that neuropeptide Y is a key modulator of body weight and that neuropeptide Y receptor antagonists may be useful anti-obesity agents.

Neuropeptide Y mediates its physiological effects via interaction with at least six distinct G protein-coupled receptors, designated Y_1, Y_2, Y_3, Y_4, Y_5 and Y_6.(Marsh et al. 1998; Michel et al. 1998; Wyss et al. 1998; Iyengar et al. 1999).

Central administration of peptide analogues of neuropeptide Y has been used to study the physiological roles of neuropeptide Y, especially the role of the peptide in feeding behavior. Kanatani et al. (1999) demonstrated an anorexogenic effect against NPY induced feeding in rats by an Y_1 antagonist with high selectivity and potency for the Y_1 receptor. Central administration of peptides that activate the Y_5 receptor, peptide YY (PYY), PYY(3-36), [Leu^{31},Pro^{34}]NPY, and human pancreatic polypeptide elicit feeding, whereas central administration of central administration of peptides that are inactive at the Y_5 receptor do not elicit feeding (Gerald et al. 1996). While other neuropeptide Y analogues or fragments interacts with several neuropeptide Y receptors, [D-Trp^{32}]NPY is a completely selective Y_5 agonist that elicits feeding at relatively high doses (Gerald et al. 1996). Peptides with activity on the Y_1 receptor produce conflicting effects on feeding. DesAA$^{10–17}$ [Cys$^{7–21}$,Pro^{34}]NPY is a Y_1 receptor agonist with a potency equivalent to neuropeptide Y, but does not elicit feeding after central administration (Kirby et al. 1995).

Schaffhauser et al. (1997) described inhibition of food intake after intracerebroventricular injection of neuropeptide Y Y_5 receptor antisense oligodeoxynucleotides in rats.

Criscione et al. (1998) reported inhibition of food intake in rats by an Y_5 receptor antagonist under vari-

ous conditions. Several neuropeptide Y antagonists were synthesized which inhibit food intake in rodents and may be used as anorectic agents (Matthews et al. 1997; Kask et al. 1998; Parker et al. 1998; Shigeri et al. 1998; Wieland et al. 1998; Zarrinmayeh et al. 1998).

Besides the stimulation of feeding, NPY and PYY show several other activities: influence on gastric motility (Chen et al. 1997), antisecretory effects in the gastrointestinal tract (Souli et al. 1997), central and peripheral control of the cardiovascular system (Lew et al. 1996; Tadepalli et al. 1996; Hudspith and Muglani 1997; McCloskey et al. 1997), effects on renal function (Blaze et al. 1997; Bischoff et al. 1997), antinociceptive effects (Broqua et al. 1996), or release of ACTH (Small et al. 1997). NPY receptor agonists show anxiolytic effects in rat conflict tests (Britton et al. 1997); neuropeptide Y_1 antagonists have anxiogenic-like effects (Kask et al. 1996).

Goumain et al. (1998) generated a rat Y_5 clone by reverse transcription from rat brain, polymerase chain reaction and transfection to COS-7 cells. Isolated jejunal crypt and villus cells and colon epithelial cells were analyzed for the Y_5 receptor by reverse transcription and polymerase chain reaction. Inhibition of binding of [^{125}I]peptide YY in these cells by various peptides was studied.

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L.4.2.2 Receptor assay of neuropeptide Y

PURPOSE AND RATIONALE

At least six distinct G protein-coupled receptors of neuropeptide Y have been identified, designated Y_1, Y_2, Y_3, Y_4, Y_5 and Y_6.(Marsh et al. 1998; Michel et al. 1998; Bischoff and Michel 1999).

Sheikh et al. (1989) described a receptor binding assay for neuropeptide Y.

PROCEDURE

^{125}I-labeling

^{125}I-labeled NPY is prepared using carrier-free Na^{125}I and 1,3,4,6-tetrachloro-3α,α6-diphenylglycouril (Serva Heidelberg, Germany) as oxidizing agent. Synthetic porcine NPY (7 nmol) in 40 µl of sodium phosphate buffer (pH 7.4, 0.1 M) and 1 mCi of Na^{125}I in 10 µl of dilute NaOH is added to a reaction vial which has been coated with 20 µg of chlorglycouril by evaporation of 20 µl of a solution of glycouril in methylene chloride. Following incubation in ice for 5 min, 50 µl of 35% acetonitrile is added prior to HPLC.

Purification by reverse phase-high performance liquid chromatography

A prepacked Nucleosil 300-5 C_{18} cartridge is used. The chromatography is performed at 50 °C with a flow rate of 1 ml 35% acetonitrile/min. Fractions of 0.5 ml are collected into tubes containing 0.1 ml of acetic acid (0.5 M) containing 10 mg/liter bovine serum albumin. Aliquots of 10 µl are counted in a γ-counter, and fractions containing the radiolabeled peptide are pooled and stored at −20 °C.

Preparation of synaptosomal membranes

Synaptosomal membranes are prepared from the hippocampus of Danish LYY-strain landrace/Yorkshire pigs. The tissue is homogenized in 0.32 M sucrose in a Sorval Omni-Mixer at 0 °C for 5 min, followed by centrifugation (1 000 *g*, 10 min). The supernatant is removed and centrifuged (20 000 *g*, 30 min) to form the crude mitochondrial fraction. After resuspension in 3 ml of sucrose, this fraction is subfractionated on a discontinuous density gradient consisting of 0.8 M and 1.2 M sucrose (3 ml of each) in a SW 27 rotor and a Beckman ultracentrifuge (100 000 *g*, 90 min). The syn-aptosomes which concentrate at the interface between the two sucrose concentrations are collected and recentrifuged (50 000 *g*, 10 min). The resulting pellet is washed with HEPES buffer (pH 7.4, 25 mM) containing $CaCl_2$ (2.5 mM) and $MgCl_2$ (1 mM), centrifuged (50 000 *g*,

10 min), and resuspended in HEPES buffer. The protein concentration is determined using the Bio-Rad protein assay with bovine serum albumin as standard. The membrane preparation is diluted in binding buffer to a protein concentration of 2 g/liter and stored at −80 °C.

Receptor binding assay

The binding buffer is a HEPES buffer (pH 7.4, 25 mM) containing $CaCl_2$ (2.5 mM) and $MgCl_2$ (1 mM), bovine serum albumin (10 g/liter), and bacitracin (0.5 g/liter). The incubation mixture consists of 0.5 ml of membrane suspension, diluted with binding buffer to a protein concentration of 200 mg/liter, 0.05 ml of unlabeled peptide, and 0.5 ml of ^{125}I-labeled peptide (40 000 cpm). After 1 h of incubation, triplicate aliquots of the incubation medium are transferred to polypropylene tubes (0.4×4.5 cm), containing 200 µl of ice-cold buffer. Membrane-bound, radiolabeled peptide is separated from the free peptide by centrifugation (7 500 *g*, 2 min) in a Beckman Microfuge B. The supernatant is aspirated, and the tube and pellet are gently washed with buffer (0.5 ml). The tip of the tube is the cut off and counted in the γ-counter.

EVALUATION

The specific binding is calculated as the difference between the amount of ^{125}I-labeled peptide bound in the absence (total binding) and presence (nonspecific binding) if 10 µM unlabeled peptide.

The concentration dependence of the receptor binding is determined by incubating the membranes with increasing concentrations of radiolabeled peptide in the absence or presence of unlabeled peptide.

MODIFICATIONS OF THE METHOD

Gehlert et al. (1996) recommended [^{125}I][Leu^{31},Pro^{34}] peptide YY as selective radioligand for the neuropeptide Y Y_1 receptor and for human pancreatic polypeptide 1 receptors.

Robin-Jagerschmidt et al. (1998) investigated the ligand binding site of neuropeptide Y at the rat Y_1 receptor by construction of mutant receptors and [^{3}H]NPY binding studies.

Dumont et al. (1998) investigated the respective distribution of neuropeptide Y Y_1, Y_2, Y_4, and Y_5 receptor subtypes in rodents (rat and mouse), guinea pig, and primates (marmoset and vervet monkey and human) brains, representing three orders of mammals.

Primus et al. (1998) measured guanyl 5'(γ[^{35}S]-thio)-triphosphate binding to NPY receptor-activated G-proteins in adult rat brain sections in order to determine the neuroanatomical distribution of NPY receptor subtypes.

Wyss et al. (1998) administered various doses of synthetic neuropeptide Y agonists intracerebroventricularly to rats in order to establish dose-response curves and to estimate ED_{50} values of feeding. These values were compared with binding affinities (IC_{50}) for rat NPY receptor subtypes Y_1, Y_2, Y_4, and Y_5 *in vitro*. Mouse fibroblast cell lines (LMTK−) were stably transfected with the rat Y_1, Y_2 and the Y_4 receptor, whereas human embryonic kidney HEK-293 cells were used for transfection with the rat Y_5 receptor.

Parker et al. (1998) determined the agonist and antagonist potency of neuropeptide Y and various analogues for cloned human and rat neuropeptide Y receptors expressed in CHO or 293 cells.

Wieland et al. (1998) studied the subtype selectivity of a nonpeptide Y_1 receptor antagonist using membranes from rat hypothalamus, from SK-N-MC (neuroblastoma) cells and from SMS-KAN (neuroblastoma) cells; human Y_1 receptor stably expressed in baby hamster kidney (BHK) cells, rat Y_1 receptor expressing human embryonic kidney (HEK) 293 cells, human Y_2 receptor stably expressed in BHK cells, rat Y_4 stably transfected in BHK cells, and human Y_5 receptor stably transfected in HEK 293 cells.

Savontaus et al. (1998) measured expression of preproneuropeptide Y mRNA in the arcuate nucleus and preprocorticotropin-releasing factor mRNA in the paraventricular nucleus by *in situ* hybridization technique after short and long-term treatment with a β_3-adrenoreceptor agonist.

Bioassays have been used for characterization and classification of neuropeptide Y receptors (Hedge et al. 1995; Pheng and Regoli 1998; Dumont et al. 2000a,b). Some isolated organs appear to be "monoreceptor" systems, e.g., the rat tail artery (Gicquiaux et al. 1996), the rabbit saphenous vein (Pheng and Regoli 1998; Feletou et al. 1999) or the isolated perfused kidney of the rat (Hedge et al. 1995) for the Y_1 receptor. The dog saphenous vein, the rat vas deferens and the rat colon can be used for the Y_2 receptor, the rat colon for the Y_4 receptor, and the rabbit ileum for the Y_5 receptor (Pheng et al. 1997).

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L.4.3 Orexin

L.4.3.1 General considerations on orexin

Orexin-A and orexin-B are 33- and 28-residue peptides, also called hypocretins, which were originally isolated from rat hypothalamus (Sakurai et al. 1998). Both peptides are derived from a 130 amino acid precursor, prepro-orexin, which is encoded by a gene localized to chromosome 17q21 in humans. These peptides are located predominantly in the hypothalamus and locus coeruleus (Evans et al. 1999), but are also found elsewhere in the brain, and in the spinal cord (Smart 1999; Van den Pol 1999). The orexins have a broad range of physiological functions, including the control of feeding and energy metabolism (Dube et al. 1999; Mondal et al. 1999; Sakurai 1999), modulation of neuro-endocrine function (Date et al. 1999), regulation of the sleep-wake cycle (Piper et al. 2000) stress and anxiety, behavioral activities (Ida et al. 1999), cardiovascular (Shirasaka et al. 1999; Chen et al. 2000), sexual and reproductive functions (Pu et al. 1998; deLecea and Sutcliffe 1999; Tamura et al. 1999). Food consumption is dose-dependently increased after intracerebroventricular infusion of orexin A and orexin B to rats which can be suppressed by a NPY Y_1 antagonist (Jain et al. 2000). Food intake could inhibited by central injection of an anti-orexin antibody in fasted rats (Yamada et al. 2000). Novak et al. (2000) found an increase of insulin secretion after subcutaneous injection of orexins to rats.

Orexin antagonists are potential anti-obesity drugs (Parker 1999).

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L.4.3.2 Receptor assay of orexin

PURPOSE AND RATIONALE

The peptides orexin A and orexin B bind both to two receptors, orexin-1 (OX_1) and orexin-2 (OX_2), although orexin B has a low affinity for OX_1 (Sakurai et al. 1998).

PROCEDURE

Orexin OX_1 and OX_2 receptors are produced by polymerase chain reaction from foetal and adult brain cDNA libraries, using primers located across the start and stop codons. The receptors are subcloned into the pCDN vector (with neomycin resistance) and transfected into CHO cells using lipofectamine (Life Technologies). Clones are selected using 400 µg/ml G418 (Life Technologies) and single clones are produced by limiting dilution cloning.

CHO-OX_1 and CHO-OX_2 cells are routinely grown as monolayers in MEM-Alpha medium supplemented with 10% fetal calf serum and 400 µg/ml G418 and maintained under 95% O_2/5% CO_2 at 37 °C. Cells are passaged every 3–4 days.

Synthetic human orexin A is ^{125}I-labeled at Tyr17 by chloramine-T oxidation in the presence of $Na^{125}I$ (2 000 Ci/mmol). Monoiodinated peptide is purified by C^{18} reverse-phase HPLC (Takigawa et al. 1995). Stable transfectant CHO cell lines expressing human OX_1R or OX_2R are each seeded onto 12-well plates at a density of 3×10^3 cells per well. After an overnight culture, medium is discarded and cells are incubated at 20 °C for 90 min with binding buffer (HEPES-buffered saline/5% bovine serum albumin) containing 10^{-10} M [^{125}I]orexin A plus designated concentrations of unlabeled competitors. Cells are then washed three times with ice-cold phosphate-buffered saline and lysed in 0.1 N NaOH. Cell-bound radioactivity is determined by a γ-counter.

EVALUATION

Data are expressed as the percentage of saturably bound reactivity in the absence of nonradioactive peptide. For each experiment each value is determined in duplicate, and the results are expressed as the means ±standard errors of at least 3 separate experiments.

MODIFICATIONS OF THE METHOD

Smart et al. (1999) studied the pharmacology of recombinant orexin receptors using the fluorescence imaging plate recorder (FLIPR). CHO-OX_1 or CHO- OX_2 cells were seeded into black walled clear base 96 well plates at a density of 20 000 cells per well in MEM-Alpha medium supplemented with 10% fetal calf serum and

400 µg/ml G418 and cultured overnight. The cells were then incubated with MEM-Alpha medium containing the cytoplasmatic calcium indicator, Fluo-3AM (4 µM) and 2.5 mM probenecid at 37 °C for 60 min. The cells were washed four times with, and finally resuspended in, Tyrode's medium containing 2.5 mM probenecid and 1% gelatin, before being incubated for 30 min at 37 °C with either buffer alone (control) or buffer containing various signal transduction modifying agents. The plates were then placed into an FLIPR (Molecular Devices, U.K.) to monitor cell fluorescence ($\lambda = 488$ nM, $\lambda = 540$ nM) (Sullivan et al. 1999) before and after the addition of orexin A or orexin B.

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L.4.3.3 Radioimmunoassay for orexin

PURPOSE AND RATIONALE

A radioimmunoassay for orexin A has been developed by Mitsuma et al. (2000).

PROCEDURE

Synthetic orexin A is conjugated on an equal weight basis to BSA using glutaraldehyde. New Zealand white rabbits are immunized with an emulsion of this conjugate in one ml of water and complete Freund's adjuvant (1 : 2, v/v) which is injected into the foot pad at intervals of 3 weeks. Blood is withdrawn one week after each injection, and the presence of anti-orexin is checked by radioimmunoassay.

Radioiodination of orexin A is performed with the chloramine T method. The radioiodinated materials are chromatographed on Sephadex G-25, eluted with 0.01 M phosphate buffer (pH 7.4), and collected in 1.0 ml fractions. The first peak is orexin A-I^{125}.

For determination of tissue concentrations, brain tissues of rats are dissected into the hypothalamus, cerebral cortex, thalamus, striatum, hippocampal formation, brain stem and cerebellum.

For the extraction of orexin A, the freshly obtained tissues are weighed and placed in 5.0 ml acid-acetone, homogenized and centrifuged.

For the double antibody radioimmunoassay, 0.1 ml of standard or samples, 0.1 ml of antibody (1 : 1 000), 0.1 ml orexin A I^{125}, and 0,5 ml buffer are incubated for 24 h at 4 °C. Then 0.1 ml of the second antibody solution is added and incubated again for 24 h at 4 °C. The samples are centrifuged, the supernatants decanted and radioactivity counted in the precipitates.

EVALUATION

Bound/total count is calculated and standard curves versus synthetic orexin A are established.

MODIFICATIONS OF THE METHOD

Using separate radioimmunoassays for orexin A and orexin B, Mondal et al. (1999) determined their distribution in microdissected nuclei of the diencephalon and brainstem of rats.

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L.4.4 Galanin

L.4.4.1 General considerations on galanin

Galanin is a neuropeptide of 29 amino acids in length (30 amino acids in humans) which was first isolated from porcine intestine (Tatemoto et al. 1983) and is localized mainly in the mammalian CNS (Skofitsch and Jacobowitz 1985; Melander et al. 1986), but also in

other organs (Baltazar et al. 2000). Central administration of galanin increases food intake in satiated rats (Crawley et al. 1990). Conversely, reduction of central galanin levels by antisense oligonucleotide techniques (Akabayashi et al. 1994) or central administration of galanin receptor antagonists (Leibowitz and Kim 1992) decreases food intake. The activity of galanin in the hypothalamus is modulated by metabolic hormones and by the ingestion of nutrients (Wang et al. 1998).

These data suggest the use of galanin receptor antagonists as anti-obesity agents.

Physiological actions of peripherally administered galanin include contraction of smooth muscle (Ekblad et al. 1989; Ahtaridis et al. 1998; Korolkiewicz et al. 1998; Niiro et al. 1998), inhibition of glucose-stimulated insulin release (McDonald et al. 1985; Leonhardt et al. 1989), influence on learning and memory behavior (McDonald and Crawley 1997; McDonald et al. 1998a,b; Gleason et al. 1999; Ögren et al. 1999; Zachariou et al. 1999), antinociceptive activity in rats with experimentally induced neuropathy (Burazin and Gundlach 1998; Ma and Bisby 1999; Yu et al. 1999; Kerr et al. 2000; Wang et al. 2000).

Galanin has been found to influence secretion of growth hormone (Ottlecz et al. 1988; Murakami et al. 1987), LH (Todd et al. 1998), luteinizing hormone (Finn et al. 1998), and prolactin (Koshiyama et al. 1987; Cai et al. 1998; Wynick et al. 1998), to inhibit dopamine release from the median eminence (Nordström et al. 1987), to influence ACh release from rat brain (Fisone et al. 1987; Kasa et al. 1998), to inhibit norepinephrine release from the hypothalamus (Tsuda et al. (1989) and to modulate 5-HT_{1A} receptors in the ventral limbic cortex of the rat (Diaz-Cabiale et al. 2000).

A fragment of the galanin precursor protein, galanin message-associated peptide (GMAP), is present in dorsal root ganglion cells and influences the spinal nociceptor flexor reflex in rats (Xu et al. 1996).

A galanin-like peptide, named GALP, was isolated by Ohtaki et al. (1999) from porcine hypothalamus.

Various **galanin receptor antagonists**, such as galantide (Lindskog et al. 1992; Sahu et al. 1994; Arletti et al. 1997; Ceresini et al. 1998), and other compounds (Bartfai et al. 1991; Pramanik and Ögren 1992; Kask et al. 1995; Xu et al. 1995; Kakuyama et al. 1997; Pooga et al. 1998; Koegler et al. 1999; Park and Baum 1999; Katoh and Ohmori 2000; Kisfalvi et al. 2000) were synthesized and tested.

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L.4.4.2 Receptor assay of galanin

PURPOSE AND RATIONALE

Galanin mediates its physiological effects via interaction with at least three G protein-coupled receptors, designated GAL1, GAL2, and GAL3 receptor (Wang and Parker 1998; Branchek et al. 2000).

The first known galanin receptor GAL1 has been isolated from the human Bowes melanoma cell line (Habert-Ortoli 1994). Human GAL1 contains 349 amino acids with the structure of a G protein-coupled receptor. A rat GAL1 homologue, cloned from Rin14B cells, contains 346 amino acids (Parker et al. 1995). Human GAL1 mRNA has been detected by northern blot analysis in fetal brain and small intestinal tissue, and also by reverse transcriptase-polymerase chain reaction in the human gastrointestinal tract from the esophagus to the rectum. Rat GAL1 mRNA has been detected by northern blot analysis in the brain, spinal cord and Rin14B cells. Human and rat GAL1 share similar binding profiles in [^{125}I]galanin binding assays (Sullivan et al. 1997).

The rat GAL2 receptor has been cloned and characterized by Wang et al. (1997), Ahmad et al. (1998), the mouse GAL2 receptor by Pang et al. (1998) and the human GAL2 receptor by Bloomquist et al. (1998) and Fathi et al. (1998). Unlike GAL1, mRNA encoding rat GAL2 is widely distributed in all tissues examined including the brain and peripheral tissues. Likewise, the human GAL2 receptor is detectable by RT-PCR in several central and peripheral tissues. Rat GAL1 and GAL2 share similar pharmacological profiles in that they possess high affinity for full-length and N-terminal fragments of galanin.

Rat and human GAL3 receptors were described by Kolakowski et al. (1998), Smith et al. (1998). Human and rat GAL3 share similar profiles in [^{125}I]galanin receptor binding assays.

Lee et al. (1999) reported the isolation of a cDNA clone named GPR54 which encodes a G-protein coupled receptor related to the galanin receptors.

PROCEDURE

Preparation of the radioligand ^{125}I-galanin is performed by iodination of galanin at room temperature by the chlor-amine-T method. Galanin, 10 µg, in 20 µl 0.05 M sodi-um phosphate buffer (pH 7.5) and 20 µl chloramine-T (5 mg/ml) are added in a batch containing 2 mCi Na^{125}I (245 mCi/ml). The reaction is terminated by adding 100 µl of a solution of sodium metabisulfite (1.2 mg/ml). The reaction mixture is transferred onto a column packed with SP-Sephadex C25, equilibrated with a solution of 100 µg/ml BSA, then washed and equilibrated with 0.05 M sodium phosphate buffer (pH 5.0). The excess of Na^{125}I is first eluted with 0.05 M sodium phosphate buffer (pH 5.0), while the iodinated galanin is eluted as a single peak with 0.05 M sodium phosphate buffer (pH 8.1). Fractions corresponding to a peak of ^{125}I-ga-lanin are pooled, the pH adjusted to 6.0 with acetic acid, and the aliquots stored at −18 °C until use.

Male Sprague-Dawley rats are sacrificed and their brains quickly removed. The hypothalamus is dissected for **preparation of membranes**. The tissue is homogenized (10% mass/vol) in 0.32 M sucrose buffered with 5 mM HEPES (pH 7.4). The homogenate is diluted 10-fold and centrifuged at 1 000 *g* for 10 min. The supernatant is centrifuged at 10 000 *g* for 45 min, and the pellet is resuspended in 5 mM HEPES-buffered Krebs-Ringer solution (pH 7.4).

Displacement experiments are carried out with various test compounds in a final volume of 400 µl HEPES/Krebs-Ringer solution, 0.05% BSA (pH 7.4), containing 1 mM ^{125}I-galanin and 70–100 µg membrane preparation. Samples are incubated for 30 min at 37 °C. Incubation is terminated by the addition of 10 ml ice-cold HEPES/Krebs-Ringer solution, followed by rapid filtration over Whatman GF/B filters, precoated for 5–6 h in 0.3% polyethyleneimine solution. Specific binding is defined as that displaceable by 1 mM rat galanin1–29.

EVALUATION

Bound/total count is calculated and displacement curves versus the standard are established.

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L.4.5 Adipsin

L.4.5.1 General considerations on adipsin

Proteins of the alternative complement pathway are secreted by adipose tissue (Choy et al. 1992; Peake et al. 1997). **Adipsin (complement D)** was the first to be cloned from an adipose tissue cell line and shown to be synthesized and secreted by adipose tissue (Cook et al. 1987; Flier et al. 1987; Johnson et al. 1991; White et al. 1992). Adipsin is markedly suppressed in *ob/ob, db/db,* monosodium glutamate-induced obese mice, in obese JCR:LA-cp rats and in cafeteria-fed rats (Rosen et al. 1989; Dugail et al. 1990; Shillabeer et al. 1992; Spurlock et al. 1996), and is regulated by glucocorticoids, retinoic acid, sympathomimetic drugs and insulin (Kitagawa et al. 1989; Spiegelman et al. 1989; Lowell and Flier 1990; Lowell et al. 1990; Moustaid et al. 1990; Antras et al. 1991; Miner et al. 1993). In contrast to rodents, adipsin increases with adiposity in humans and in response to feeding and is decreased during fasting, cachexia and lipatrophy (Napolitano et al. 1994).

Adipsin is required for the synthesis of **acylation stimulating protein (ASP)**, a protein implicated in fat metabolism (Sniderman and Cianflone 1994; Cianflone et al. 1999; van Harmelen et al. 1999). ASP is produced by the cleavage of C3a by carboxypeptidase and is highly expressed by mature adipocytes. The synthesis of C3a from C3 requires complement factor B and adipsin. Plasma ASP increases with meals and facilitates the synthesis and storage of triglycerides. Consistent with its role as a mediator of lipogenesis, ASP deficiency increases postprandial fatty acid levels and decreases weight gain and trigylceride synthesis in mice (Murray et al. 1999, 2000).

Several other factors, such as adiponectin (Takahashi et al. 2000) are involved in the function of adipose tissue acting as target as well as an endocrine organ (Ahima and Flier 2000).

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L.4.5.2 Adipsin expression in mice

PURPOSE AND RATIONALE

Adipsin expression at the protein and mRNA levels was studied in mice (Flier et al. 1987; Spiegelman et al. 1989; Lowell et al. 1990; Dugail et al. 1990) and rats (Miner et al. 1993).

PROCEDURE

Various strains of mice (CD-1 mice, C57BL6 *ob/ob* mice, C57BL/Ks misty diabetes (m db/m db) mice, and mice with monosodium glutamate induced obesity are treated with drugs, e.g., ephedrine (1 g/kg chow) and caffeine (1.4 g/kg chow) mixed in the diet. The effects of these regimens on fat pad weight, specific mRNAs in white epididymal and brown adipose tissue and serum adipsin concentrations are assessed.

RNA is extracted from epididymal adipose tissue, brown intercapsular adipose tissue, 3T3-F422A adipocytes and isolated rat adipocytes by the guanidium-cesium chloride technique. Total RNA is denaturated, electrophoresed in 1.5% agarose, transferred to nylon filters, and hybridized to random primed adipsin, uncoupling protein (UCP), or actin cDNAs (Flier et al. 1987).

Serum adipsin and adipsin released by 3T3-F442A cultured adipocytes are assessed by RIA. Mouse adipsin purified from Chinese hamster ovary cells stably transfected with an adipsin expression vector is iodinated using the Bolton-Hunter reagent. The labeled protein is separated from unincorporated ^{125}I using G-50 Sephadex chromatography. The first rabbit polyclonal antibody is raised to purified mouse adipsin overexpressed using a baculovirus expression system. The assay is carried out in PBS, pH 7.4, supplemented with $CaCl_2$ (0.1 g/liter), $MgCl_2$ (0.1 g/liter), and BSA (0.1%). Serum adipsin is assessed in a dilution of 1 : 1 000–1 : 5 000 and in culture media at a dilution of 1 : 100. After adding tracer, standards, and serum, the tubes are mixed and the first antibody is added at a dilution of 1 : 800. After an overnight incubation, the bound and unbound tracer are separated using goat anti-rabbit immunoglobulin C fixed to heat killed staphylococcus at a ratio 1 : 1.2.

EVALUATION

Standard curves are generated using mouse adipsin purified from stably transfected CHO cells. Serial dilutions of standards, serum, and culture media are compared.

MODIFICATIONS OF THE METHOD

Dugail et al. (1989) showed that, in sharp contrast with genetically obese mice, adipsin mRNA is not suppressed in genetically obese Zucker rats.

Platt et al. (1994) found that a tissue-specific transcription factor that regulates adipsin expression is less active in the adipose tissue of obese animals.

Napolitano et al. (1994) determined concentrations in blood and rates of adipsin secretion by adipose tissue in humans using a two-monoclonal 'sandwich' ELISA assay.

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Chapter M
Anti-atherosclerotic activity[1]

[1] With contributions for this edition by H.-L. Schäfer.

M.1 Induction of experimental atherosclerosis

M.1.0.1 General considerations

Experimental atherosclerosis was first successfully induced in rabbits by Saltykow (1908) and Ignatowski (1909). During the following years, various scientists found that dietary cholesterol was the responsible stimulus for development of atherosclerosis. Other species are also susceptible to diet-induced atherosclerosis (Reviews by Kritchevsky 1964; Hadjiinky et al. 1991). A unifying hypothesis of the pathogenesis of atherosclerosis has been proposed by Schwartz et al. (1991).

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M.1.0.2 Cholesterol-diet induced atherosclerosis in rabbits and other species

PURPOSE AND RATIONALE

Rabbits are known to be susceptible to hypercholesterolemia and arteriosclerosis after excessive cholesterol feeding. Therefore, this approach has been chosen by many authors to study the effect of potential anti-arteriosclerotic drugs.

PROCEDURE

Several modifications of the protocol have been described. Usually, male rabbits from an inbred strain, e.g., white New Zealand, at an age of 8–10 weeks are used. Body weight variation should be as low as possible. At the beginning of the experiment, blood is withdrawn from the marginal ear vein for determination of total cholesterol, total glycerides, and blood sugar. Groups of 10 animals are used for treatment with drugs or as controls. The rabbits are switched from commercial food to a diet supplemented with 0.3–2% cholesterol and kept on this regimen for a period of 10–12 weeks. One group is kept on normal diet. During and at the end of the experiment blood is taken for analysis. Usually, cholesterol and triglyceride levels increase several-fold over the original values.

The animals are sacrificed and the thoracic aorta is removed, cleaned of surrounding tissues, and longitudinally cut and opened for fixation with formaldehyde. The tissue is stained with oil red. The percentage of the intimal surface covered by the oil red positive lesions is calculated with a computerized planimeter. In animals fed a normal diet, the aorta does not show any staining, whereas in cholesterol-fed rabbits the aorta shows severe atherogenic lesions.

EVALUATION

Data are expressed as means ± standard deviation. Statistical evaluation is performed by Dunnett's or Scheffé's test. A p-value of < 0.05 is regarded as statistically significant.

MODIFICATIONS OF THE METHOD

Shore and Shore (1976) studied two **different strains of rabbits** (New Zealand White and Dutch Belt) as models of hyperlipoproteinemia and atherosclerosis.

Studies of Kritchevsky et al. (1989) on experimental atherosclerosis in **rabbits** fed **cholesterol-free diets** revealed a greater influence of animal protein and of partially hydrogenated soybean oil on development of atherosclerosis than plant protein and unsaturated soybean oil.

Cockerels (Tennent et al. 1960) and **turkeys** (Simpson and Harms 1969) are very susceptible to cholesterol feeding and develop marked hypercholesterolemia in rather short periods. Atherosclerosis could also be induced in cockerels by high doses of estrogen without atherogenic diet (Caldwell and Suydam 1959).

Spontaneous arteriosclerosis in **pigeons** has been described by Clarkson and Lofland (1961).

The **Japanese sea quail** (*Coturnix coturnix japonica*) is highly susceptible to the rapid development of severe experimental atherosclerosis (Day et al. 1975, 1977, 1979, 1990; Chapman et al. 1976).

Out of 13 strains of **mice**, Roberts and Thompson (1976) selected the C57BR/cdJ and the CBA/J strain und used these strains and their hybrids as models for atherosclerosis research.

Paigen et al. (1987) described quantitative assessment of atherosclerotic lesions in mice. After 14 weeks on an atherogenetic diet C57BL/6J female mice had aortic lesions at each of the coronary arteries, at the junction of the aorta to the heart and in scattered areas of the aortic surface. The lesions increased after 9 months of atherogenic diet. Methods of evaluating the number and size of lesions were compared including sizing with a microscope eyepiece grid and computer-assisted planimetry.

Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice were described by Paigen et al. (1990).

Yamaguchi et al. (1993) found that addition of 10% linoleic acid to a high-cholesterol diet enhanced cholesterol deposition in the aorta of male ICR strain mice.

In **rats** hypercholesterolemia can be induced by daily administration by gavage of 1 ml/100 g body weight of a cocktail containing in 1 l peanut oil: 100 g cholesterol, 30 g propylthio-uracil, and 100 g cholic acid over a period of 7 days. The test compounds are administered simultaneously with the cocktail (Fillios et al. 1956; Lustalot et al. 1961).

Inoue et al. (1990) induced experimental atherosclerosis in the rat carotid artery by balloon de-endothelialization and atherogenic diet. A balloon catheter was introduced into the rat's carotid arteries from the iliac arteries and the endothelium was denuded.

The **hamster** is susceptible to atherosclerosis. Nistor et al. (1987) fed male hamsters a hyperlipidemic diet consisting of standard chow supplemented with 3% cholesterol and 15% commercial butter for 12 months. Serum total cholesterol doubled after 3 weeks and attained a 17-fold value after 10 months. Up to 6 months, smooth muscle cells in the intima and media of the aorta as well as endothelial cells began to load with lipids. After 10 months the affected zones looked like human atherosclerotic plaque with huge cholesterol crystal deposits, calcium deposits and necrosis.

Especially the hybrid hamster strain Bio™ F_1B (Bio Breeders Fitchburg, MA, USA) is more susceptible to dietary induced atherosclerosis than other strains (Kowala et al. 1991). Early atherosclerotic lesions can be induced within a 3-months-feeding of a cholesterol/butter enriched diet. In these animals simvastin dose-dependently inhibited the development of hyperlipidemia and the plaque formation by cholesterol synthesis inhibition The histopathological examination of the aortas showed that the cholesterol/butter fed F_1B hamster developed atherosclerotic lesions and functional changes in the aorta which are closely related to man (Schäfer et al. 1999).

Soret et al. (1976) studied the diet-induced hypercholesterinemia in the diabetic and non-diabetic Chinese hamster.

Beitz and Mest (1991) used cholesterol-fed **guinea pigs** to study the antihyperlipemic effects of a potentially anti-atherosclerotic drug.

Malinow et al. (1976) recommended the **cynomolgus monkey** as a model for therapeutic intervention on established coronary atherosclerosis.

This species was used by Hollander et al. (1978) to study the development atherosclerosis after a cholesterol and fat enriched diet.

Beere et al. (1992) described experimental atherosclerosis at the carotid bifurcation of the cynomolgus monkey by a cholesterol-enriched diet.

Eggen et al. (1991) studied the progression and the regression of diet-induced atherosclerotic lesions in aorta and coronary arteries on **rhesus monkeys**.

Howard (1976) recommended the **baboon** as model in atherosclerosis research because of the similarity of cholesterol metabolism and composition of the lipoproteins to man.

Kushwaha et al. (1991) determined the effect of estrogen and progesterone on plasma cholesterol concentrations and on arterial lesions in ovarectomized and hysterectomized baboons fed a high-cholesterol/high-saturated-fat diet.

Blaton an Peeters (1976) reported studies on the **chimpanzee**, the **baboon** and the **rhesus macacus** as models for atherosclerosis.

Ming-Peng et al. (1990) studied high density lipoproteins and prevention of experimental atherosclerosis in **tree shrews** (*Tupaia belangera yunalis*). In contrast to rabbits, no increased lipid deposition in aortic intima after cholesterol feeding was found in tree shrews.

CRITICAL ASSESSMENT OF THE METHOD

Diet-induced hypercholesterolemia is useful only for detection of agents interfering with the adsorption, degradation and excretion of cholesterol. Agents interfering with cholesterol biosynthesis are less probable to be detected.

The use of normal adult **marmosets**, a species with a lipoprotein profile similar to that of man, may be an alternative (Crook et al. 1990; Baxter et al. 1992).

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M.1.0.3 Hereditary hypercholesterolemia in rats

A strain of genetically hypercholesterolemic rats (RICO) was described by Müller et al. (1979). In contrast to Zucker-rats, these animals are normotriglyce-ridemic and non-obese. The hypercholesterolemia of the RICO rat is related to a decreased rate of catabolism of chylomicrons and LDL, but more specifically to an excessive production of these two types of lipoproteins. This strain has been proposed to study hypolipidemic drugs, particularly those designed to decrease the plasma concentrations of chylomicrons and LDL. Hypolipidemic effects of β-cyclodextrin were found in this model (Riottot et al. 1993).

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M.1.0.4 Hereditary hyperlipemia in rabbits

Watanabe et al. (1977, 1980) described a strain of rabbits with hereditary hyperlipemia (WHHL rabbit) which has been used by several scientists to study development of atherosclerosis, as well as for histological and functional changes of the aorta. At the age of 10–14 months homozygous animals exhibit an atheromatous plaque, distributed heterogeneously over the luminal surface of the aorta. Serum cholesterol is increased up to 400–600 mg/dl. The increased levels of LDL have been studied in detail (Kita et al. 1981, 1982).

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M.1.0.5 Transgenic animals

Several transgenic animals as disease model were created during the last decade, mice (Stoltzfus and Rubin 1993), rats and rabbits.

The widely used model is the Apo E knockout mouse originally created by Nubuyo Maeda, University of North Carolina, Chapell Hill, NC. These Apo E knockout mice have spontaneously elevated plasma cholesterol levels, and develop atherosclerosis even on regular chow within 3–4 months. The time dependent progression of atherosclerosis leads to lesions similar in histopathology to those observed in humans. This

animal model is used as background for atherosclerosis research and target validation.

Walsh et al. (1989) and Rubin et al. (1991) integrated human apolipoprotein A-I gene in transgenic mice resulting in an increase of HDL levels.

Linton et al. (1993) described the development of transgenic mice expressing high levels of human apolipoprotein B48 and human apolipoprotein B100 which are considered to be atherogenic.

Transgenic mice lacking apolipoprotein E showed severe hypercholesterolemia and atherosclerosis (Plumpp et al. 1992; Zhang et al. 1992).

Overexpression of apolipoprotein E in transgenic mice reduced plasma cholesterol and triglyceride levels, prevented hypercholesterolemia and inhibited the formation of fatty streak lesions (Harada et al. 1996).

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M.1.0.6 Evaluation of endothelial function in rabbits with atherosclerosis

PURPOSE AND RATIONALE

Cholesterol feeding of rabbits impairs the endothelium-dependent relaxation evoked by acetylcholine in the aorta. This phenomenon can be used to study the influence of vasodilators as well as the prevention by ACE-inhibitors (Becker et al. 1991).

PROCEDURE

Male white New Zealand rabbits weighing 3–4 kg receive a hypercholesterolemic diet containing 0.25 to 1% cholesterol and 3% coconut oil. Rabbits of the same weight receiving standard diet serve as controls. After several weeks, the serum cholesterol levels are increased from 30–40 mg/dl in the control group up to 900–1 000 mg/dl in the cholesterol-fed group. At the end of the treatment period, the animals are sacrificed by intravenous injection of sodium pentobarbital and a complete autopsy is performed.

Intact proximal parts of the thoracic aorta are sectioned into 2 mm wide rings, cut off to strips, and suspended at 2 g gauge in 25 ml organ chambers filled with a buffer solution of 37 °C comprising 113.8 mM NaCl, 20 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 5.5 mM glucose being gassed with a 95% O_2/5% CO_2 mixture to achieve a pH of 7.4. After 2 h, when a stable contractile tone is established, norepinephrine is added at a final concentration of 1×10^{-8} M, which produces a stable submaximum isotonic contraction. Then, acetylcholine is added in 10 fold incremental doses from 1×10^{-8} M up to final concentrations of 10^{-5} M. Relaxation of aortic strips is assessed as percentage decrease in contraction. Acetylcholine induced concentration-dependent relaxation is greatly impaired in aorta rings from cholesterol-fed rabbits whereas contractions to norepinephrine are only slightly diminished.

EVALUATION

The data are expressed as mean ±SEM and compared by Student's *t*-test for unpaired data.

MODIFICATIONS OF THE METHOD

Verbeuren et al. (1986, 1990) and Tagawa et al. (1991) used a bioassay for the determination of EDRF (Rubanyi et al. 1985). The donor thoracic aorta segments (3 cm long) are mounted horizontally in perfusion chambers filled with physiological salt solution which contains indomethacin (3×10^{-6} M) at 37 °C; the solution is gassed with a mixture of 20% O_2, 5% CO_2, and 75% N_2. The aortas (control and atherosclerotic tissue) and a piece of glass tubing are perfused continuously with the same solution at 3 ml/min. The perfusate is dripping directly onto a segment (5 mm long) of either a control or an atherosclerotic abdominal aorta from which the endothelium was mechanically removed. The detector abdominal aortas are mounted vertically over two hooks and the development of isometric tension is monitored continuously. The initial tension of the detector aortas is set at 8 g. The perfusate of each donor aorta is analyzed both on a control and on an

atherosclerotic detector preparation: the order of this double analysis is selected at random. A control and an atherosclerotic donor aorta are always analyzed in parallel and thus on the same detector tissue. Drugs are added to the perfusion medium. Before the start of the experiments, the tissues are allowed to equilibrate for 45 min in the perfusion chambers. By means of a three-way system, placed at the outlet of the donor aortas, atropine (10^{-6} M) is added to the perfusate to block any muscarinic contractile effect of acetylcholine on the detector tissue. Noradrenaline (2×10^{-6} M) is infused into the perfusate via this 3-way system causing a sustained contraction in the detector tissue.

After the stabilization of the contraction to noradrenaline, the highest dose of acetylcholine (1.6×10^{-8} mol) is added to the solution for perfusion of the tubing in order to confirm no change in the contractile response to noradrenaline in the detector tissues. Moreover, the perfusate from the control and atherosclerotic donor aortas under basal conditions does not significantly affect the contraction of the detector tissues. The tissues are allowed to equilibrate and then increasing doses of acetylcholine (0.01 to 160×10^{-10} mol) are injected into the perfusion medium close to the donor aortas. Relaxation is less pronounced in the detector tissues when atherosclerotic aortas are stimulated with acetylcholine indicating that the cholinergic agent causes a smaller release of EDRF from atherosclerotic donor aortas than from control donor aortas. The degree of the arteriosclerotic lesion can be assessed by this functional assay.

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M.1.0.7 Intimal reactions after endothelial injury

PURPOSE AND RATIONALE

Several attempts have been made to induce intimal injury in animals which is followed by proliferation and formation of fatty streaks similar to the alterations found in human atherosclerosis. One approach is the "balloon catheterization".

PROCEDURE

Male New Zealand White rabbits weighing 2.0–3.5 kg or male Sprague-Dawley rats weighing 350–400 g are used. An embolectomy catheter (Edwards Laboratories, size 4 French for rabbits, size 2 French for rats) is introduced into the right femoral artery under surgical anesthesia and passed to the aortic arch. After inflation with room air, the catheter is withdrawn to the iliac bifurcation, deflated and removed.

The incorporation of [^{3}H]thymidine into DNA of rabbit or rat aorta is measured 48 h after balloon catheterization. Animals are sacrificed 45 min after intravenous injection of [^{3}H]thymidine. Intima-media is prepared from whole aorta by scraping with blunt forceps. The specific activity of ^{3}H in DNA is determined after extraction of the DNA in the washed tissue homogenate using hot dilute perchloric acid. DNA is assayed by the diphenylamine method. ^{3}H Incorporation is measured in a liquid scintillation counter.

For histological examination, rabbits are injected 2 weeks after balloon catheterization intravenously with heparin (500 U/kg) and then sacrificed. Fixative (2% glutaraldehyde in 0.15 M phosphate buffer, pH 7.4) is infused at a constant pressure of 100 mm Hg via a carotid cannula and the aorta is pressure fixed *in situ* for 60 min. Intimal proliferation is quantified in the upper abdominal aorta, in the lower abdominal aorta and half-way between these points.

EVALUATION

[^{3}H]Thymidine incorporation and intimal proliferation is compared between drug treated animals and controls.

MODIFICATIONS OF THE METHOD

DeCampli et al. (1988) studied the effects of various drugs on accelerated myointimal proliferation in canine veno-arterial allografts by histological methods. An 8-cm length of femoral vein was removed, reversed, divided, and sewn end-to-end into carotid or femoral arteries of a recipient dog.

Berkenboom et al. (1989) induced experimental atherosclerosis in **canine** and **porcine** coronary arteries by endothelial denudation followed by a high-cholesterol diet.

Kawata et al. (1990) described the detection of regenerating cells in the aorta after ballooning by immunocytochemical demonstration of the thymidine analogue 5-bromo-2'-deoxyuridine.

Manderson et al. (1990) described changes in vascular reactivity of carotid arteries in rabbits following endothelial denudation.

Bocan et al. (1991) tested an ACAT inhibitor and selected lipid-lowering agents for anti-atherosclerotic activity in iliac-femoral and thoracic aortic lesions. Atherosclerotic lesion comparable in composition to human fatty streaks were induced by chronic endothelial denudation in the iliac-femoral artery inserting a sterile, indwelling, 18-cm nylon filament with a diameter of 200 µm into the lumen of the right femoral artery in hypercholesterolemic New Zealand White rabbits. Naturally occurring fatty streaks developed in the thoracic aorta following cholesterol feeding. The effect of treatment on lesion regression was evaluated in the iliac-femoral artery, while changes in the lesion progression were evaluated in the thoracic artery.

Davies et al. (1993) performed right common carotid artery bypass grafting using the ipsilateral external jugal vein in New Zealand White rabbits and studied the response to endothelin-1 in normolipidemic and hyperlipidemic animals.

Groves et al. (1993) studied platelet adhesion and thrombus formation in a **porcine** model of balloon angioplasty.

ACE inhibitor treatment reduced the neointima formation after endothelial denudation in the carotid artery of **rats** using a balloon catheter (Farhy et al. 1992; Linz and Schölkens 1992; Linz et al. 1993, 1994).

Lyle et al. (1995) tested the effect of inhibitors of factor X_a or platelet adhesion, heparin, and aspirin on platelet deposition in an atherosclerotic rabbit model of angioplastic injury. Acute ^{111}indium-labelled platelet deposition and thrombosis were assessed 4 h after balloon-injury in femoral arteries subjected to prior endothelial damage (air desiccation) and cholesterol supplementation (1 month).

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M.2 Influence on lipid metabolism

M.2.0.1 General considerations

Elevated lipid levels, especially hypercholesterolemia, result from increased absorption from the gut or enhanced endogenous synthesis. Therefore, two ways are feasible to reduce hyperlipidemia: to block endogenous

synthesis or to decrease absorption. Both factors can be evaluated in normal animals without artificial diets. Clinically used lipid lowering compounds like fibrates can be tested in this way, being investigated with additional tests. Earlier attempts to interfere with endogenous cholesterol synthesis resulted in accumulation of other sterols than cholesterol (Holmes 1964). The most extensively studied approach to inhibit cholesterol biosynthesis is with HMG-CoA reductase inhibitors. Inhibition of other enzymes of cholesterol biosynthesis, such as lanosterol 14α-methyl demethylaseα and squalene synthetase, is considered. The inhibition of cholesterol absorption by ACAT-inhibitors is a widely followed approach. Furthermore, the interruption of bile acid recirculation resulting in reduction of LDL cholesterol is being used.

Protective effects of calcium antagonists against experimental arteriosclerosis acting mainly on other mechanisms than lipid metabolism have been claimed by various authors but the clinical relevance is still questionable (Kjeldsen and Stender 1989; Fronek 1990; Knorr and Kazda 1990; Fleckenstein-Grün et al. 1992).

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M.2.0.2 Hypolipidemic activity in rats

PURPOSE AND RATIONALE

Hyperlipoproteinemia with increased concentrations of cholesterol- and triglyceride-carrying lipoproteins is considered to be the cause of arteriosclerosis with its dual sequelae of thrombosis and infarction. Lipoproteins are divided into 6 major classes: chylomicrons, chylomicron remnants, VLDL (very low density lipoproteins), IDL (intermediate density lipoproteins), LDL (low density lipoproteins), and HDL (high density lipoproteins). HDL promotes the removal of cholesterol from peripheral cells and facilitates its delivery back to the liver. Therefore, increased levels of HDL are desirable. On the contrary, high levels of VLDL and LDL promote arteriosclerosis. LDL, especially in its oxidized form, is taken up by macrophages via a scavenger mechanism. Therefore, anti-arteriosclerotic drugs should reduce VLDL and LDL and/or elevate HDL.

PROCEDURE

Groups of 10 male Wistar rats weighing 180–200 g are used. They are given once daily in the morning over a period of 8 days the test compounds or the standard in various doses ranging from 1 to 100 mg/kg via stomach tube in a volume of 5 ml/kg. The control group is given the solvent (e.g., PEG 400) only. Body weight of each animal is registered at the beginning and at the end of the experiment. Twenty hours prior to the experiment, food but not water is withdrawn. On the morning of the first day, blood samples are taken under light ether anesthesia by retroorbital puncture. Then, the first dose is applied. During the whole period, the animals have free access to food and water. Twenty hours prior the end of the experiment, food is again withdrawn and blood samples are taken by retroorbital puncture. Immediately thereafter, the animals are sacrificed and the liver removed, blotted free from blood and weighed. Samples of liver are frozen in liquid nitrogen and stored at –25 °C for lipid analysis. The blood samples are centrifuged for 2 min at 16 000 *g*. Total cholesterol and total glycerin as a measure of triglycerides are determined in each blood sample.

To estimate the serum lipoproteins, the serum of each rat group is pooled. The serum lipoproteins are separated by means of a preparative ultracentrifuge (e.g., KONTRON TGA 65, Rotor TFT 456).

The separation of fractions VLDL, LDL, HDL, and of the subnatant of HDL is carried out as follows:

VLDL	native density of the serum (1.006), 16 h at 40 000 rpm,
LDL	density range from 1.006 to 1.04, 18 h at 40 000 rpm,
HDL	density range from 1.04 to 1.21, 18 h at 40 000 rpm,
Subnatant of HDL	density > 1.21.

The density is adjusted by addition of a calculated amount of NaBr solution.

Cholesterol is determined using Boehringer Mannheim test combinations by the CHOD-PAP high performance method (Siedel et al. 1983) and triglycerides by means of an enzymatic assay (Eggstein and Kreutz 1966; Wahlefeld 1974). The method of Lowry et al. (1951) is applied to determine protein content.

Frozen samples of liver are thawed and extracted with chloroform/methanol 2 : 1 (v/v) in a Teflon Potter-Elvehjem homogenizer. The extracts are purified according to Folch et al. (1957). Solvents of aliquots of the extracts are evaporated for determinations of cholesterol and triglycerides.

EVALUATION

Average values of body weight, cholesterol and triglycerides are expressed as percentage of initial values for each group at the end of the experiment. Statistical differences between the controls and the treatment groups are evaluated by covariance analysis.

CRITICAL ASSESSMENT OF THE METHOD

Studies with normocholesterinemic animals are faced with the difficulty that starting cholesterol levels are relatively low, and to achieve significance for lowering of cholesterol levels requires large groups of animals.

MODIFICATIONS OF THE METHOD

Similar tests can be performed in various species, e.g.:

- Male NMRI mice, weighing 25–30 g,
- male New Zealand obese (NZO) mice, weighing 35–40 g,
- male Syrian golden hamsters, weighing 70–100 g,
- male Pirbright guinea pigs, weighing 200–250 g,
- male miniature pigs, weighing 14–22 kg.

To study long term effects, the experiments are extended for 4 weeks or 3 months.

Schurr et al. (1976) proposed high volume screening procedures for hypobetalipoproteinemic activity in rats.

März et al. (1993) described fast lipoprotein chromatography as a new method of analysis for plasma lipoproteins.

Adipokinetic actions of several hormones in slices of perirenal adipose tissue of various species were determined by Rudman et al. (1963).

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M.2.0.3 Hypolipidemic activity in Syrian hamsters

PURPOSE AND RATIONALE

The Syrian hamster (*Mesocricetus auratus*) is a widely used animal to study the effects of drugs and diet on lipoprotein metabolism. Several in human approved lipid lowering drug like HMG-Co A reductase inhibitors, or cholestyramine lower plasma cholesterol in hamster. The lipoprotein and bile acid metabolism of the hamster is closer to human than the lipoprotein and bile acid metabolism of rats and mice (Bravo et al. 1994; Suckling et al. 1991; Kris-Etheron and Dietschy 1997). The hamster has in contrast to pig, rat and mouse CETP (Cholesteryl Ester Transfer Protein) activity, similarly as seen in humans (Ha and Barter 1982, 1986; Ahn et al. 1994).

Increase in plasma cholesterol can be easily induced by adding small, physiological amounts of cholesterol to the diet (0.05–0.01 weight%). Additional saturated fat like coconut oil (5–10 weight%) has synergistic effects for induction of hyperlipidemia. (Kowala et al. 1991) A stable hyperlipidemia with a human like lipoprotein pattern can be induced in hamster within 2–3 weeks by adding 10 weight% coconut butter and 0.2% cholesterol into the diet. Hamster HDL cholesterol can be easily measured after precipitation of VLDL+LDL cholesterol with phoshotungstic acid/$MgCl_2$ (Weingand and Daggy 1990, 1991). LDL and HDL cholesterol can also be measured directly with Kits from Roche Diagnostics. A complete separation of all lipoprotein fractions can be done by FPLC on Superose 6 columns (Pharmacia) according the method of März et al. (1993).

PROCEDURE

Male Syrian hamsters weighing 95–125 g at the start of the experiment are randomly assigned to form groups of 6 animals each. For each group a different diet is prepared: Controls receive powdered lab chow only, for the other groups test drug or standard (cholestyramine 0.1 to 2%) is added to the diet. After 1 week on these diets, the animals are anesthetized with diethyl ether, a blood sample is taken from the superior vena cava and the liver is removed and weighed. Microsomes are prepared by ultracentrifugation from the livers.

Preparation of plasma lipoprotein profiles

The blood is centrifuged at 600 *g* for 5 min and the plasma removed. The plasma is analyzed for total cholesterol using a colorimetric enzymatic assay (Merck, CHOD-iodine, BDH). The cholesterol content of HDL is determined using a precipitation kit (Merck, BDH).

Quantification of high-density-lipoprotein cholesterol by differential precipitation

Weingand and Daggy (1990) compared the validity of differential precipitation with Mg^{2+}-phosphotungstate for quantification of plasma high-density-lipoprotein cholesterol with ultracentrifugal flotation.

As precipitating agent, 0.55 mmol/l phoshotungstic acid and 25 mmol/l magnesium chloride is added. Cholesterol is determined spectrophotometrically with an enzymatic cholesterol reagent containing microbial cholesterol esterase, in a Hitachi 705 clinical chemistry analyzer.

Fast lipoprotein chromatography

März et al. (1989, 1993) developed fast lipoprotein chromatography for rapid and quantitative analysis of lipoprotein fractions.

Fast lipoprotein chromatography is carried out with a chromatography system from Kontron consisting of two Model 420 pumps, a Model 432 variable-wavelength detector, and a Model 450 data system. Without using a pretreatment, 20 µl of plasma are applied to a 300-mm Superose column 6 equilibrated with 100 mmol/l Na_2HPO_4, pH 7.4, and 200 mmol/l NaCl. Lipoproteins are detected on-line at 500 nm after post-column derivatization with CHOD-PAP cholesterol reagent (Boehringer Mannheim, Germany). The column eluate and the cholesterol reagent are mixed in a motor-driven microchamber attached to the column outlet, and the mixture is then passed through a "knitted" capillary (20–22). The flow rate of the reagent is 70 µl/min; the run time is 80 min. Under these conditions, it takes the column eluate 2 min to pass the derivatization capillary. VLDL-C, LDL-C and HDL-C are calculated on the basis of relative peak areas and total cholesterol.

Tissue preparation

The liver is homogenized in a KCl (0.104 M) solution containing NaF (50 mM) using a glass Teflon homogenizer. The homogenate is centrifuged at 30 000 *g*, 5 °C for 25 min. The supernatant is then centrifuged at 100 000 *g*, 5 °C for 60 min to pellet the microsomes, which are resuspended in potassium phosphate buffer (50 mM) containing NaF (50 mM) adjusted to pH 7.4.

Determination of enzyme activities in microsomal fractions

Protein for all the following assays is determined using the method described by Bradford (1976).

HMG-CoA reductase activity is determined by quantifying the conversion of [^{14}C]HMG-CoA to [^{14}C]mevalonic acid lactone, based on the method described by Ingebritsen and Gibson (1981). ACAT activity in liver microsomes and intestinal cell homogenates is determined by the method described by Suckling et al. (1982), measuring the incorporation of [^{14}C]oleoyl-CoA into cholesteryl oleate. The cholesterol 7α-hydroxylase activity in the microsomes is quantified by determination of the percentage conversion of [^{14}C]cholesterol to 7α-hydroxy[^{14}C]cholesterol. 3 mg of microsomal protein is diluted to a volume of 5.3 ml with a potassium phosphate buffer (50 mM, pH 7.4) containing NaF (50 mM), cysteamine (31.4 mM), glucose-6-phosphate (12.7 mM) NADP (1.4 mM) and [^{14}C]cholesterol (0.22 µCi, 55 Ci/mol). After a short pre-incubation (5 min at 37 °C), the reaction is started by the addition of glucose-6-phosphate dehydrogenase suspension (7 µg). The reaction is stopped 1 h later by adding methanol (5 ml). The cholesterol and 7α-hydroxy-cholesterol are extracted into a mixture of chloroform and methanol (2 : 1, v/v, 2 × 5 ml). The product and substrate are separated by thin-layer chromatography on silica gel eluting with toluene/ethyl acetate (3 : 7, v/v). The radioactive regions corresponding to cholesterol and 7α-hydroxy-cholesterol are scraped off the plates and quantified by liquid scintillation counting.

EVALUATION

Dose-response curves for standard and test drug are established using the control data as zero.

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M.2.0.4 Triton-induced hyperlipidemia

PURPOSE AND RATIONALE

The systemic administration of the surfactant Triton to mice or rats results in a biphasic elevation of plasma cholesterol and triglycerides (Frantz and Hinkelman 1955; Garattini et al. 1958, 1961; Holmes 1964; Tamasi et al. 1968).

PROCEDURE

Male Sprague Dawley or Wistar rats weighing 200–350 g are starved for 18 h and then injected intravenously with 200 mg/kg Triton WR 1 339 (isooctyl-polyoxyethylene phenol). Serum cholesterol levels increase sharply 2–3 times after 24 h (phase I). The hypercholesterolemia decreases nearly to control levels within the next 24 h (phase II). The test drugs employed or the solvent for the controls are administered simultaneously with the Triton injection or 22 h thereafter. Serum cholesterol analyses are made 6, 24, and 48 h after Triton injection. The mechanism of the Triton-induced hypercholesterolemia in phase I is thought to be due to increased hepatic synthesis of cholesterol through the ability of Triton to interfere with the uptake of plasma lipids by the tissues. Drugs interfering with cholesterol biosynthesis were shown to be active in phase I, while drugs interfering with cholesterol excretion and metabolism were active in phase II.

EVALUATION
Mean values ± standard deviation are calculated for each group and time interval and compared statistically with the controls.

CRITICAL ASSESSMENT OF THE METHOD
The method employing Triton hypercholesterolemia is rather simple and rapid for detection of compounds interfering with the synthesis and excretion of cholesterol. Since the test is rather artificial, the results have to be validated by other methods.

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M.2.0.5 Fructose induced hypertriglyceridemia in rats

PURPOSE AND RATIONALE
Rats switched from a diet low in carbohydrates and high in protein to a high intake of fructose, develop an acute hypertriglyceridemia. Compounds are tested for inhibition of this phenomenon.

PROCEDURE
Male Sprague Dawley rats weighing 200–250 g are fed over a period of one week a diet enriched in protein with reduced carbohydrate content, e.g., Altromin® C1 080 or C1 009. Groups of 10 animals are treated for 3 days daily with the test compound or the standard (clofibrate 100 mg/kg) or the vehicle (polyethylene glycol) by oral gavage. From the second to the third day water is withheld for a period of 24 h. Immediately afterwards, the animals are offered 20% fructose solution ad libitum for a period of 20 h. After this time which is also 20 h after the last application of the test compound, the animals are anesthetized with ether and 1.2 ml blood is withdrawn by retroorbital puncture. The blood is centrifuged for 2 min at 16 000 *g*. Total glycerol is determined in the serum according to Eggstein and Kreutz (1966) and total cholesterol according to Richterlich and Lauber (1962).

EVALUATION
The average values of total glycerol of the treated groups are compared with the control group using Student's *t*-test.

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M.2.0.6 Intravenous lipid tolerance test in rats

PURPOSE AND RATIONALE
Intravenous injection of a lipid emulsion results in an increase of triglycerides in serum. The lipolytic activity can be determined by measuring lipid elimination.

PROCEDURE
Male Wistar rats weighing 200–240 g are treated daily with various doses of the test compound or the vehicle over a period of 5 days. On the fifth day, two hours after the last administration of the test compound, the animals are anesthetized with 125 mg/kg sodium hexobarbital i.p. Then they are injected intravenously with 2 ml/kg of a 10% lipid emulsion (Intralipid® Vitrum, Hausmann AG, St. Gallen, Switzerland). Prior to the injection and 10, 20, 30, and 40 min thereafter blood is withdrawn by retro-orbital puncture for determination of triglycerides.

EVALUATION
Peak levels as well as elimination constant and half life are determined.

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M.2.0.7 Influence on lipoprotein-lipase activity

PURPOSE AND RATIONALE

Postheparin plasma lipolytic activity is performed by at least two lipase activities: hepatic triglyceride lipase and extrahepatic lipoprotein lipase. Hypothyroid rats show a selective decline of postheparin plasma hepatic triglyceride lipase (Murase and Uchimura 1980). For patients with familial lipoprotein lipase deficiency a rapid diagnostic test was developed (Gotoda et al. 1991). The method was used to study the influence of drugs on lipoprotein lipase activity (Tsusumi et al. 1993).

PROCEDURE

Male Wistar rats weighing 180–200 g receive either various single doses or one daily dose of test compounds over a period of several days. Blood samples are drawn from the tail vein into tubes containing 1 mg EDTA/ml. The animals are then injected with 100 U/kg heparin via the tail vein and blood samples are collected 5 min later. Plasma samples are used to determine lipoprotein lipase and hepatic triglyceride lipase activity.

Lipoprotein lipase activity in postheparin plasma is measured using glycerol tri[1-^{14}C]oleate as substrate and selective blocking of hepatic lipase activity with antiserum to rat hepatic lipase. Hepatic triglyceride lipase activity in postheparin plasma is obtained by subtracting lipoprotein lipase activity from total plasma lipase activity.

EVALUATION

The dose-related increase of plasma lipoprotein lipase activity and the time course after drug administration are evaluated.

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M.2.0.8 Influence on several steps of cholesterol absorption and formation

PURPOSE AND RATIONALE

Cholesterol levels in the body result from two sources: absorption from the gut and endogenous *de novo* synthesis. Certain natural and synthetic compounds inhibit cholesterol absorption and reduce plasma cholesterol levels in experimental animals and are therefore of potential pharmacologic interest in the treatment of hypercholesterinemia. Cholesterol absorption can be decreased by inhibition of acyl coenzyme A:cholesterol acyltransferase (ACAT). Inhibition of cholesterol resorption results in a compensatory increase of hepatic 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) activity reductase activity which can be decreased by (HMG-CoA) reductase inhibitors. Harwood et al. (1993) studied the pharmacologic consequences of cholesterol absorption inhibition: alteration in cholesterol metabolism and reduction in plasma cholesterol concentration induced by a synthetic saponin β-tigogenin cellobioside.

PROCEDURE

Male golden Syrian hamsters, weighing 100–120 g, are housed in a reversed light-dark cycle room (light between 3:00 P.M. and 3:00 A.M.) and receive a cholesterol poor diet and water ad libitum 1 week prior to use. The animals are assigned to groups of 6 animals each and are given free access to water and chow that contains the compounds to be tested in appropriate concentrations for 4 days. At 9:00 A.M. of the fourth day of the study, the animals receive a 1.0 ml oral bolus of liquid hamster diet containing 15 mg of [^{3}H]cholesterol (2.25 μCi) and 7.5 mg cholic acid for determining cholesterol absorption. At 9:00 A.M. of the fifth day (peak of the diurnal cycles of HMG-CoA reductase and cholesterol 7α-hydroxylaseα activities) animals are anesthetized with pentobarbital and blood samples are obtained by cardiac puncture for determining plasma cholesterol and triglyceride levels, and cholesterol absorption.

Livers are removed, weighed, rinsed in 4 °C saline, and apportioned for determining hepatic cholesterol levels, hepatic HMG-CoA reductase, cholesterol 7α-hydroxylase activities, hepatic LDL receptor concentration, and cholesterol absorption.

Hepatic microsomes for measurement of hepatic HMG-CoA reductase activity and cholesterol 7α-hydroxylase activity are prepared according to Harwood et al. (1984) and Junker and Story (1985). For measurement of HMG-CoA reductase activity, 0.5 g liver are immediately homogenized at 4 °C in 1 ml TEDK buffer (containing 50 mM Tris (pH 7.5), 1 mM EDTA, 5.0 mM dithiothreitol, and 70 mM KCl) using 15 strikes in a Dounce homogenizer. For measurement of cholesterol 7α-hydroxylase activity, 0.5 g liver pieces are immediately homogenized at 4 °C in 1 ml PEDSKF buffer (containing 40 mM phosphate (pH 7.4), 5 mM EDTA, 5 mM DTT, 250 mM sucrose, 50 mM KCl, and 50 mM KF) using 20 strokes of a Dounce homogenizer. Homogenates are first centrifuged at 4 °C for 20 min at 10 000 *g* and the resultant supernatant is then centrifuged at 4 °C for 90 min at 178 000 *g*. The resulting microsomal pellets are resuspended in either 1.0 ml TEDK per g liver (HMG-CoA reductase determination) or 1 ml PEDSKF per g liver (cholesterol 7α-hydroxylase determination) by 5 strokes of a Potter-Elvehjem pestle and are stored frozen in liquid nitrogen.

For measurement of hepatic HMG-CoA reductase activity, 50 µg of microsomal protein is incubated for 30 min at 37 °C in a final volume of 75 µl of TEDK buffer containing 3.4 mM $NADP^+$, 30 mM glucose-6-phosphate, 66.7 µM [^{14}C]HMG-CoA (10 cpm/pmol), 15 000–20 000 cpm [^{3}H]mevalonate (0.6–1.2 Ci/mmol) as an internal standard, and 68 mM EDTA to prevent conversion of mevalonate to phosphomevalonate during incubation. After incubation, 10 µl of 6 M HCl are added to terminate the enzyme reaction and to convert the newly formed mevalonate to mevalonolactone. The mevalonolactone is then separated from unreacted substrate by silica gel thin-layer chromatography. After development in toluene-acetone 1 : 1, the region of the chromatogram corresponding to $R_f = 0.4–1.0$ is removed, immersed in liquid scintillation fluid, and counted using a dual channel $^3H/^{14}C$ program. HMG-CoA reductase activity is expressed as pmoles of mevalonate formed from HMG-CoA per min of incubation at 37 °C per mg of microsomal protein.

Cholesterol 7α-hydroxylase activity is measured in liver microsomes according to Junker and Story (1985) with increased specific activity of the added radiolabeled cholesterol in order to enhance the sensitivity of the method. An aliquot of 0.5 µg of microsomal protein, in a final volume of 200 µl PEDKF buffer (containing 40 mM phosphate (pH 7.4), 5 mM EDTA, 5 mM DTT, 50 mM KCl, and 50 mM KF), is mixed in a 16 × 125 mm test tube with 100 µl PEDKF containing 0.5 µCi [^{14}C]cholesterol, 0.5 mg Triton WR-1 339, and 5 µl acetone. The mixture is incubated in a covered, shaking water bath at 37 °C for 1 h to allow exogenously added [^{14}C]cholesterol to equilibrate with the unlabeled microsomal cholesterol pool.

The enzymatic reaction is initiated by addition of 250 µl of 70 mM phosphate buffer (pH 7.4) containing 1.25 mM $NADP^+$, 10 mM glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase, and 10 mM β-mercaptoethyleneamine. After a 30 min-incubation at 37 °C, the reaction is terminated by addition of 7.5 ml chloroform:methanol 2 : 1 and vigorously mixed for 20 s. One ml distilled water is then added and the mixtures are vigorously shaken for an additional 10 s. After centrifugation at 2 500 rpm for 5 min at 4 °C, the chloroform layer is transferred to a test tube and evaporated to dryness under nitrogen. The resultant lipid residue is dissolved in 100 µl chloroform and 20 µl aliquots are applied to silica gel thin-layer plates and developed in toluene:ethylacetate 1 : 9. The region of the chromatogram corresponding to 7α-hydroxycholesterol is removed from the plate, mixed with 10 ml of liquid scintillation fluid and assessed for radioactivity. Cholesterol 7α-hydroxylase activity is expressed as pmoles of 7α-hydroxycholesterol formed from cholesterol per min of incubation per mg microsomal protein. The substrate specific activity for each microsomal sample is calculated by dividing the total substrate radioactivity by the total amount of cholesterol present in the assay.

Hepatic LDL receptor levels are measured by enzyme immune blotting (Cosgrove et al. 1992). One hundred –150 µl of the soluble extracts containing 100–600 µg protein are adjusted to 2% SDS and 0.2 M sucrose by addition of 0.33 volumes of electrophoresis sample buffer (containing 320 mM Tris (pH 6.8), 8% SDS, and 0.8 M sucrose). Mixtures are applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5% (w/w) 0.1% SDS-containing polyacrylamide slab gel of 1.5 mm thickness. Electrophoresis is conducted at room temperature with a constant current of 15 mA/gel. Prestained molecular weight markers are included in a separate lane to monitor separation. After electrophoresis, proteins migrating into the gel are electrophoretically transferred to S&S BA85 nitrocellulose membranes at 18 °C with a con-

stant voltage of 120 V for 6–8 h in 25 mM Tris, 192 mM glycine buffer (pH 8.3), containing 20% methanol.

After transfer, the nitrocellulose paper is incubated with 100 ml of Tris buffered saline containing 3% gelatin for 30 min at room temperature with gentle shaking. After incubation, the nitrocellulose sheet is removed from the blocking solutions, immersed without rinsing into 50 ml Tris buffer solution (TBS) containing 1% gelatin and 250 µl of anti-LDL receptor peptide antiserum (final dilution 1 : 200) and incubated for 2 h at room temperature with gentle shaking. The nitrocellulose sheet is washed twice for 10 min each with TBS containing 0.05% Tween-20 and once for 10 min with TBS. The washed nitrocellulose sheet is incubated with 50 ml TBS containing 1% gelatin and 100 µl of goat anti-rabbit IgG-horseradish conjugate (final dilution 1 : 500) at room temperature for 1 h with gentle shaking. After incubation, the nitrocellulose is washed again as described above. During this final wash, 40 mg of 4-chloro-1-naphthol is dissolved in 10 ml methanol, and 50 µl cold 30% hydrogen peroxide is added to 50 ml TBS containing 1% gelatin. After draining the final TBS wash from the nitrocellulose, the two solutions are mixed and immediately added to the nitrocellulose. The mixture is incubated at room temperature with gentle shaking until the desired color development is observed. The nitrocellulose sheet is washed with running tap water for 15 min. The nitrocellulose is dried between pieces of filter paper and the intensity of color formation is quantitated by reflectance densitometry using a suitable instrument (e.g., Hoefer Scientific Instruments GS300 Transmittance/Reflectance Scanning Densitometer). Color intensity, which is a linear function of the number of LDL receptors in the analyzed fraction, is expressed in terms of mm peak height (arbitrary reflectance units).

Intestinal cholesterol absorption is estimated from the sum of radioactivity present in the liver and plasma 24 h after an oral bolus of [^{3}H]cholesterol. For assessment of hepatic radioactivity, 1.0-g liver pieces are placed in 50-ml polypropylene tubes and incubated with 2.5 ml of 2.5 M KOH for 2 h at 75 °C. After allowing the saponification mixture to cool to room temperature, 5.0 ml of 80% ethanol, 0.1 ml [^{14}C]cholesterol (40 000 dpm, 15 dpm/nmol) as carrier and extraction standard and 10 ml of hexane are added. Tubes are capped and shaken vigorously for 1 min. Mixtures are permitted to stand to allow phase separation, and are shaken vigorously for an additional minute. Mixtures are the centrifuged at 1 000 *g* for 5 min. Duplicate 3.0-ml aliquots of each hexane layer are removed, transferred to 20-ml liquid scintillation vials, mixed with 10 ml liquid scintillation fluid, and counted using a dual channel ^{3}H/^{14}C program.

After correction for recovery losses, hepatic radioactivity is calculated based on total liver weights. For assessment of total plasma radioactivity, 200-µl aliquots of plasma are added to 20 ml liquid scintillation vials and decolorized by addition of 25 µl of hydrogen peroxide. Ten ml aqueous scintillation fluid is added and the mixture assessed for radioactivity. Total radioactivity in the plasma is calculated based on the assumption that hamsters possess approximately 4.0 ml plasma per 100 g body weight. The degree of cholesterol absorption is expressed as a percentage of the total radioactivity administered that is present in the liver plus plasma 24 h after bolus administration.

For determination of **hepatic cholesterol concentration**, a 200-µl aliquot of the liver homogenate obtained after Dounce homogenization in TEDK buffer is mixed with 350 µl of TEDK buffer in a test tube. Chloroform-methanol 2 : 1 (7.5 ml) is then added and the suspension is vigorously mixed for 1 min. The sample is allowed to stand for 20 min, then 1 ml deionized water is added and the mixture vigorously shaken for 10 s. The mixture is centrifuged at 6 000 rpm for 15 min at room temperature. The chloroform-methanol layer is removed, placed in a 13 × 100 mm test tube, and evaporated to dryness under nitrogen at 37 °C. The lipid residue is resuspended in 200 µl isopropanol and the cholesterol concentration determined according to Assmann et al. (1983). Total hepatic cholesterol concentrations are expressed as µg cholesterol per mg hepatic protein.

For **plasma lipid and lipoprotein determinations**, blood plasma samples are treated either with EDTA or heparin to prevent clotting and are then centrifuged at 2 500 rpm for 30 min at 4 °C. The resultant plasma is removed and analyzed for cholesterol and triglyceride content using commercially available kits. HDL-cholesterol concentration is measured directly after precipitation with phoshotungstic acid/$MgCl_2$ of apoB-containing lipoproteins (Assmann 1983). Non-HDL cholesterol concentration (LDL + IDL + VLDL) is estimated by subtraction.

EVALUATION

Inhibition of cholesterol resorption results in a decrease of hepatic cholesterol and a compensatory increase of hepatic HMG-CoA reductase activity resulting from de novo cholesterol synthesis and an increase of hepatic LDL receptor levels.

MODIFICATIONS OF THE METHOD

Ogishima and Okuda (1986) described an improved method for assay of cholesterol 7α-hydroylase activityα. Cholesterol 7α-hydroxylation is performed in liver microsomes utilizing cholesterol as substrate. 7α-Hydroycholesterol is converted by the action of cholesterol oxidase into 7α-hydro-4-cholesten-3-one having an intense absorption at 240 nm.

Hylemon et al. (1989) reported a modified HPLC-spectrophotometric method for measuring cholesterol 7α-hydroxylase activity by using a C-18 reverse-phase column to separate 7α-hydroxy-4-cholesten-3-one and 4-cholestene-3-one and by adding 7β-hydroxycholesterol to each reaction mixture as an internal recovery standard. This method measures simultaneously cholesterol 7α-hydroxylase activity using endogenous cholesterol and exogenous [4-^{14}C]cholesterol as substrate.

Princen and Meijer (1990) measured cholesterol 7α-hydroxylase activity in cultured rat hepatocytes.

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M.3
Inhibition of cholesterol biosynthesis

M.3.1
General considerations on cholesterol biosynthesis

The following steps are involved in cholesterol biosynthesis:

- HMG-CoA synthase (hydroxymethylglutaryl-coenzyme A synthase)
- forming **hydroxymethylglutaryl-CoA** from acetyl-CoA and acetoacetyl-CoA
- HMG-CoA reductase (hydroxymethylglutaryl-coenzyme A reductase)
- forming **mevalonic acid** from hydroxymethylglutaryl-CoA.

Inhibition of cholesterol synthesis on this step is used at present successfully for therapy (see below)

- Mevalonate kinase forming **5-phospho-mevalonic acid** from mevalonic acid,
- Phospho-mevalonate kinase forming **5-pyrophospho-mevalonic acid** from 5-phospho-mevalonic acid,
- Pyrophospho-mevalonate decarboxylase forming **3-isopentyl-pyrophosphate** from 5-pyrophospho-mevalonic acid,
- Isopentyl-pyrophosphate isomerase forming **3,3-dimethyl-pyrophosphate** from 3-isopentyl-pyrophosphate,
- Dimethylallyl-transferase forming in a two step process **geranyl pyrophosphate** from 3,3-dimethyl-pyrophosphate, and
- **farnesyl pyrophosphate** from geranyl pyrophosphate,
- Squalene synthetase forming **squalene** from farnesyl pyrophosphate,
- Squalene epoxidase forming **2,3 oxidosqualene** from squalene,
- 2,3-Oxidosqualene cyclase forming **lanosterol** from 2,3 oxidosqualene.

Following the formation of lanosterol (4,4,14α-trimethylcholesta-8(9),24-dien-3β-ol), a series of enzyme reactions is required to produce cholesterol (Bae et al. 1999):

- to 4,4,14α-trimethylcholesta-8(9)-en-3β-ol,
- to 4,4-dimethylcholesta-8(9),14-dien-3β-ol,
- to 4,4-dimethylcholesta-8(9)-en-3β-ol,
- to cholesta-8(9)-en-3β-ol,
- to cholesta-7-en-3β-ol,

- to cholesta-5,7-dien-3β-ol (7-dehydrocholesterol), and finally
- to cholesta-5-en-3β-ol (cholesterol) by 7-dehydrocholesterol reductase.

Besides **inhibition of HMG-CoA reductase** (detailed description below) other approaches to inhibit biosynthesis of cholesterol are reported:

- **inhibition of HMG-CoA synthase** (Goldstein and Brown 1990; Miller et al. 1980; Greenspan et al. 1987; Grayson and Westkaemper 1988);
- **inhibition of squalene synthase** (Biller et al. 1991a,b; Oehlschlager et al. 1991; Baxter et al. 1992; Ciosek et al. 1993; Ness et al. 1994; Harris et al. 1995; Chan et al. 1996; Dufresne et al. 1996; McTaggart et al. 1996; Amin et al. 1997; Sliskovic and Picard 1997; Rosenberg 1998; Vaidya et al. 1998; Hiyoshi et al. 2000; Ugawa 2000);

  Squalene synthase plays an important role in sterol biosynthesis by catalyzing the head-to-head condensation of two molecules of farnesylpyrophosphate. The enzyme is a single 47 000 Da polypeptide that is bound to the subcellular membranes of the endoplasmatic reticulum in yeast and mammalian liver;
- **inhibition of squalene epoxidase** (Horie et al. 1990, 1991; Moore et al. 1992; Ryder 1992);

  Squalene epoxidase catalyzes the conversion of squalene to (3*S*)-2,3-oxidosqualene, an essential step in the biosynthesis of sterols in mammals, plants, and microorganisms.

  A simplified squalene epoxidase assay based on HPCL separation and time-dependent UV/visible determination of squalene was published by Grieveson et al. (1997);
- **inhibition of 2,3-oxidosqualene cyclase** (Gerst et al. 1986; Cattel et al. 1989; Sen and Prestwich 1989; Dollis and Schuber 1994; Mark et al. 1996; Eisele et al. 1997; Morand et al. 1997; Abe et al. 1998a);

  The enzyme 2,3-oxidosqualene cyclase is of special interest due to its dual function: cyclization of 2,3-monoepoxysqualene to lanosterol and 2,3:22,23-diepoxysqualene to oxylanosterol.

  An orally active oxidosqualene:lanosterol cyclase inhibitor (Ro48-8 071) showed potent noncompetitive inhibition of bacterial squalene:hopene cyclase from *Alicyclobacillus acidocaldarius*. A tritium-labeled isotopomer of this nonterpenoid inhibitor, which possesses a benzophenone photophore, was chemically synthesized as a photoaffinity label (Abe et al. 1998b);
- **inhibition of 7-dehydrocholesterol reductase** (Amin et al. 1996);

  Deficiency of this enzyme causes a severe developmental disorder with multiple congenital and morphogenic abnormalities, the Smith-Lemli-Opitz syndrome (Waterham and Wanders 2000).

Search and development of new cholesterol-lowering drugs is continued (Abe and Prestwich 1998). Whether the efficacy and safety of these agents in man will be on the long run superior to currently available therapies remains to be determined.

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M.3.2
Determination of HMG-CoA-reductase inhibitory activity

M.3.2.1
General considerations on HMG-CoA reductase

More than 70% of the total production of body cholesterol in humans is derived from de novo synthesis. 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is the rate limiting enzyme governing cholesterol biosynthesis and the synthesis of other isoprenoids in mammalian cells (Rodwell et al. 1976). The development of HMG-CoA reductase inhibitors offers an advance in the treatment of hypercholesterolemia by interfering with the crucial step of cholesterol biosynthesis. When inhibiting hepatic HMG-CoA reductase, the inhibitors trigger an increased production of LDL receptors in the liver. As LDL receptor activity increases, more LDL is extracted from the

blood and thus the level of circulating LDL-cholesterol is reduced. Pharmacological evaluation of HMG-CoA reductase inhibitors is based on studies on the inhibition of the isolated enzyme HMG-CoA -reductase *in vitro*, on the inhibition of the incorporation of ^{14}C sodium acetate into cholesterol in isolated liver cells, and on the effect of HMG-CoA -reductase inhibitors *in vivo*. HMG-CoA reductase activity has a diurnal rhythm in liver and intestine which has to be considered for *in vivo* studies (Shapiro and Rodwell 1969; Shefer et al. 1972).

HMG-CoA reductase inhibitors have been associated with skeletal myopathy in humans and experimental animals (Flint et al. 1997). The search for other inhibitors of cholesterol biosynthesis without myotoxicity seems to be worthwhile.

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M.3.2.2 Inhibition of the isolated enzyme HMG-CoA-reductase *in vitro*

PURPOSE AND RATIONALE

For screening purposes, studies on the inhibition of HMG-CoA reductase obtained from rat liver microsomal fraction can be used (Avigan et al. 1975; Philipp and Shapiro 1979).

PROCEDURE

The inhibitory activity of the test compound on HMG-CoA reductase is estimated with soluble enzyme preparations obtained from the microsomal fraction of rat liver (Philipp and Shapiro 1979). The enzyme reaction is carried out with 50 µl partially purified HMG-CoA reductase in buffer containing 25 mM Tris, 10 mM EDTA, and 10 mM dithiothreitol at pH 7.5, 20 µl of 910 µM HMG-CoA solution containing 100 nCi (3.7 KBq) of ^{14}C-HMG-CoA and 20 µl of NADPH regenerating system (5.2×10^{-2} M glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 5.3×10^{-3} M NADP), with the actual concentration of 50 mM NADPH. The final incubation volume is 200 µl. The main reaction is preceded by 20 min preincubation with the NADPH regenerating system at 37 °C, followed by 20 min incubation at 37 °C of the completed samples with the test compound or the standard and stopped by addition of 75 µl 2 N $HClO_4$. After 60 min at room temperature, the samples are cooled in an ice-bath and neutralized by addi-

tion of 75 µl 3 N potassium acetate. Supplementing the volume with water to 500 µl, the precipitate is centrifuged and 250 µl of the clear supernatant are applied to a column (0.6 × 8.0 cm) of BIORAD AG 1-X8 (100–200 mesh). Mevalonolactone is eluted with water discarding the first 750 µl and collecting the next 3 500 µl. Five hundred µl of the eluate are used for measurement in duplicate, mixed in vials with 10 ml Quickscint (Zinsser) and measured in a liquid scintillation counter (Beckman). The assay is generally performed in triplicate. Lovastatin sodium is used as standard.

EVALUATION

The mean values with and without inhibitors are compared for the calculation of inhibition. IC_{50} values are calculated.

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M.3.2.3 Inhibition of the incorporation of ^{14}C sodium acetate into cholesterol in isolated liver cells

PURPOSE AND RATIONALE

De novo synthesis of cholesterol from labelled acetate can be measured in isolated liver cells. This synthesis can be inhibited by incubation with HMG-CoA reductase inhibitors.

PROCEDURE

Monolayers of primary cell cultures from hepatocytes of female rats or of cultures of HEP-G2 cells (human hepatoma cells) are incubated in a lipoprotein deficient medium for 1 h with various concentrations of the potential HMG-CoA reductase inhibitor or the standard (Lovastatin). Thereafter, the labelled precursor ^{14}C sodium acetate is added to the medium and the incubation continued for further 3 or 24 h. To one part of the cells an internal standard of ^{3}H-cholesterol is added and the cells are saponified by alkaline. The lipids of these saponified cells are extracted with chloroform/methanol. The lipid mixture is separated by preparative thin layer chromatography. The localization of cholesterol is identified by exposure to iodine vapor and the amount of newly synthesized ^{14}C-cholesterol is determined in a scintillation counter.

Cell protein is determined in another part of the cells. A third part of the cell culture is used to control the integrity of the cells by light microscopy and tested biologically by determination of the release of lactate dehydrogenase into the incubation medium.

EVALUATION

The amount of newly synthesized cholesterol per mg cell protein is calculated. The inhibitory capacity of the test drug is estimated against a solvent control. IC_{50} values are calculated from the results of various concentrations.

MODIFICATIONS OF THE METHOD

Chen and Kandutsch (1976) studied the effects of cholesterol derivatives on sterol biosynthesis in cultures of various cell types.

Liver specificity of inhibition of sterol synthesis is studied by *in vitro* uptake into hepatocytes and human skin fibroblasts (Scott 1990) or in slices of various tissues after oral treatment with HMG-CoA reductase inhibitors (Tsujita 1990).

Shaw et al. (1990), however, caution against the use of HEP-G2 cells which may not be the cell system of choice to demonstrate liver selectivity of inhibitors of HMG-CoA reductase.

Cultures of HEP-G2 cells have also be used to test inhibition of cytoplasmic acetoacetyl-CoA thiolase (Greenspan et al. 1989) or inhibition of squalene epoxidase (Hidaka et al. 1991).

Pearce et al. (1992) used HEP G2 cells to study the hypocholesterolemic activity of synthetic and natural tocotrienols *in vitro*.

Raiteri et al. (1997) investigated drugs acting at different steps of the mevalonate pathway on arterial smooth muscle cell proliferation. Competitive HMG-CoA reductase inhibitors dose-dependently decreased smooth muscle cell proliferation.

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M.3.2.4 *Ex vivo* inhibition of cholesterol biosynthesis in isolated rat liver slices

PURPOSE AND RATIONALE

Inhibition of cholesterol biosynthesis in rat livers can be measured in an *ex vivo* assay after oral treatment with HMG-CoA reductase inhibitors by cholesterol synthesis from labelled sodium octanoate.

PROCEDURE

Male Sprague Dawley rats weighing 110–130 g are kept on a reverse light cycle (lights 3:00 P.M. to 3:00 A.M.) for 14 days prior to use. Throughout the period of adaptation, the rats have free access to a low cholesterol diet and tap water. On the day of the experiment, the test compounds are given orally between 9:00 and 11:00 A.M. as suspensions in 0.5% methylcellulose. After one hour, the rats are sacrificed, the livers removed and transferred to chilled oxygenated Krebs-Ringer-bicarbonate buffer (pH 7.4). The livers are then chopped into 0.8-mm^2 pieces using a McIlwain tissue chopper (e.g., Brinkmann Instr., Westbury, USA) and are suspended in the same buffer. Aliquots of the suspension are pipetted, in triplicate, into culture tubes which contain [^{14}C]sodium octanoate (300 µM/l, 6.67 Ci/M). The assay volume is 1 ml. The tubes are gassed with 95% O_2/5% CO_2 for 10 s, stoppered with a serum cap, and incubated at 37 °C in a metabolic shaker at 150 oscillations/min for 90 min.

The reaction is stopped by addition of 1 ml 15% KOH in ethanol. An aliquot of the mixture is assayed for protein concentration. An internal standard [^{3}H]cholesterol (30 000 dpm) is added to determine recovery, which ranges from 70–80%. The tubes are saponified at 75 °C for 2 h and then extracted with 10 ml of petroleum ether for 30 min. The lower aqueous phase is frozen in a dry ice/alcohol mixture, and the ether phase is removed, washed with 2 ml glass-distilled water and then evaporated to dryness. The [^{14}C]cholesterol synthesized is separated by thin layer chromatography on plastic silica gel plates using chloroform as eluent. After visualization with iodine, the cholesterol spots are cut out, and radioactivity quantitated by liquid scintillation counting.

EVALUATION

Results are expressed as percentage inhibition compared to vehicle-treated control values. Using various doses, ED_{50} values of inhibition can be calculated from dose-response curves.

MODIFICATIONS OF THE METHOD

Koga et al. (1990) tested the tissue-selective inhibition of cholesterol synthesis *in vivo* by pravastin sodium in mice. Drugs were orally administered to mice 2 h prior to an intraperitoneal injection of [^{14}C]acetate. The animals were sacrificed 1 h afterwards and the cholesterol synthesis determined in various organs.

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M.3.2.5 Effect of HMG-CoA-reductase inhibitors *in vivo*

PURPOSE AND RATIONALE

A strain of rabbits with heritable hyperlipidemia, the WHHL strain, has been described by Watanabe et al. (1980, 1985, 1988). These animals develop digital xanthoma and aortic and coronary atherosclerosis already at an early age. This animal is considered to be a suitable model for the evaluation of preventive or even regressive effects of drugs on hyperlipidemia and atherosclerosis.

PROCEDURE

Male heterozygous WHHL rabbits weighing 1.8 to 2.5 kg at an age between 8 and 20 weeks are used. The animals are housed individually under standard conditions (standard rabbit diet and water ad libitum) and are allowed to accommodate 2 weeks prior to treatment. The test compounds are suspended in 0.5% methylcellulose and are administered each day orally by gavage in the afternoon to insure an increased plasma level at night, since in man HMG-CoA reductase activity has been found to be higher at night than during daytime (Shapiro and Rodwell 1969; Shefer et al. 1972) similar to the enzyme in rodents. The treatment is continued for 14 days.

Blood samples are taken in the morning without previous feeding. Two ml of blood are drawn from the outer ear vein 5 days prior to the beginning of treatment, on days 3 and 8 of treatment and 30 days after the end of treatment for the determination of biochemical parameters. In addition, 6 ml blood are drawn at the first and the last day of treatment and 10 days after the end of treatment for determination of biochemical parameters and lipoprotein profile. In order to obtain serum, blood is allowed to clot at room temperature and then centrifuged twice at 10 000 rpm.

The following biochemical parameters are determined in non-frozen samples (kept at 4 °C): total cholesterol, HDL-cholesterol, triacylglycerol, as well as creatinine, total bilirubin, alkaline phosphatase, alanine amino transferase (ALAT), aspartate amino transferase (ASAT), and γ-glutamyl transferase (γ-GT) using commercially available kits.

The separation of serum lipoproteins by gel permeation chromatography is performed according to Ha and Barter (1985). This method is particularly well suited for the metabolic studies of lipoproteins, because the elution profile can be obtained from the same sample under more gentle conditions than by sequential ultracentrifugation. According to their descending particle size, the elution profile of lipoproteins in the same fraction of density (<1.21 g/ml) shows, as the elution progresses, three major peaks which correspond to very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL), respectively.

Since the amount of serum collected from one rabbit is too low to determine the lipoprotein profile from each sample, the total lipoproteins are isolated by ultracentrifugation at a density < 1.21 g/ml and the lipoproteins thus obtained in each group are pooled two by two prior to injection onto the cross-linked agarose HR 50 column (Superose 6B). As a result, each lipoprotein profile represents the lipoprotein size distribution of an equal volume of lipoproteins obtained from two rabbits. Cholesterol and triacylglycerol concentrations are determined in each 1 ml elution fraction.

EVALUATION

The data at 5 days before beginning of treatment and of day 0 of each animal are pooled and the mean is taken as reference value. Student's paired *t*-test is used to calculate for each group the significance of difference between mean values.

MODIFICATIONS OF THE METHOD

Extension of the treatment period up to 24 weeks allows to evaluate the meanwhile apparent atherosclerotic lesions in the aorta and the coronaries by gross observation and light microscopy (Tsujita 1990).

Kasim et al. (1993) studied the effect of lovastatin on the secretion of very low density lipoprotein lipids and apolipoprotein B in the Zucker obese rat

which is basically a model for genetic hypertriglyceridemia.

Soma et al. (1993) studied the effects of HMG-CoA reductase inhibitors on carotid intimal thickening induced by placing a nonocclusive, biologically inert, soft, hollow Silastic collar around both carotid arteries in normocholesterolemic rabbits (Booth et al. 1989).

Bocan et al. (1994) assessed atherosclerotic lesion development in the thoracic artery and chronically denuded iliac-femoral artery of hypercholesterolemic New Zealand White rabbits using inhibitors of HMG-CoA reductase.

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M.4 Inhibition of cholesterol absorption

M.4.1 Inhibition of ACAT (Acyl coenzyme A:cholesterol acyltransferase)

M.4.1.1 General considerations

Acyl coenzyme A:cholesterol acyltransferase (ACAT), which catalyses the intracellular formation of cholesteryl esters, plays an important role in the intestinal absorption of cholesterol, foam cell formation within the arterial wall and VLDL production in the liver. Cholesterol is absorbed from the gut exclusively in the unesterified form, but appears in the lymph esterified with various long-chain unsaturated fatty acids. The enzyme responsible is ACAT, a microsomal enzyme, that utilizes long-chain fatty acyl coenzyme A and cholesterol as substrates. ACAT inhibitors also have potential actions beyond inhibition of cholesterol absorption. Inhibition of hepatic ACAT could reduce the production of cholesteryl esters for packaging into lipoproteins, while inhibition of ACAT in the arterial wall could reduce the deposition of cholesteryl esters in atherosclerotic lesions.

Human ACAT cDNA was cloned from a human macrophage cDNA library and expressed in an ACAT deficient line of Chinese hamster ovary cells. The cDNA, labelled K1, encoded an integral membrane protein of 550 amino acids (Chang et al. 1993). The inhibition of ACAT as treatment for hypercholesterolemia and atherosclerosis is an attractive target.

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M.4.1.2 *In vitro* ACAT inhibitory activity

PURPOSE AND RATIONALE

In vitro ACAT inhibitory activity can be determined in microsomal preparations from liver or intestine of rabbits.

PROCEDURE

Hepatic or intestinal microsomes are prepared from rabbits. Prior to sacrifice, the animals receive chow supplemented with 2% cholesterol and 10% safflower oil for 6 weeks. Each assay contains 0.2 mg of microsomal protein and fatty acid-poor bovine serum albumin (3 mg/ml) in 0.04 M KH_2PO_4 buffer, pH 7.4, containing 0.05 M KCl, 0.03 M EDTA, and 0.3 M sucrose. Drug dilutions are made in DMSO (5 µl DMSO/200 µl total incubation volume). The reaction is started by the addition of [^{14}C]oleyl CoA (50 µM, 7 dpm/pmol). After 3 min the reaction is stopped by the addition of chloroform-methanol 2 : 1. [^{3}H]Cholesteryl oleate is used as an internal standard. Lipid extracts are dissolved in chloroform, spotted on TLC plates (silica gel G) and developed in hexane-petroleum ether-acetic acid 80 : 20 : 1. Unlabeled, carrier cholesterol oleate is added to the internal standard to aid band visualization with iodine vapor. The band corresponding to cholesteryl esters is then scraped into scintillation vials and radioactivity is determined by liquid scintillation spectroscopy.

EVALUATION

For each compound four concentrations are evaluated in duplicate. IC_{50} values are determined by performing a nonlinear least-squares fit of the data to a log dose-response curve.

MODIFICATIONS OF THE METHOD

Rothblatt et al. (1977) studied ACAT activity in Fu5 rat hepatoma cells under the influence of hyperlipemic serum lipoproteins.

Mathur et al. (1981) studied ACAT activity in hepatic microsomes of cynomolgus monkeys during diet-induced hypercholesterolemia.

Einarsson et al. (1989) studied acyl-CoA:cholesterol acyltransferase activity in human liver microsomes.

Largis et al. (1989) found CL 277,082 to be a potent inhibitor of ACAT in microsomes from a variety of tissues and smooth muscle cells in culture.

Heffron et al. (1990) studied ACAT inhibition in microsomal fractions of the transformed mouse macrophage J774.

Bell et al. (1992) measured ACAT inhibition in cultured Fu5AH cells.

Field et al. (1991), Krause et al. (1993) tested the inhibition of ACAT in CaCo-2 cells.

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M.4.1.3 *In vivo* tests for ACAT inhibitory activity

PURPOSE AND RATIONALE

Most authors test the *in vivo* anti-atherosclerotic and antihyperlipemic effect of ACAT inhibitors in cholesterol-fed hypercholesterolemic animals (Balasubramaniam et al. 1990; Harris et al. 1992; Tanaka et al. 1944). Krause et al. (1993a) used the Zilversmit dual isotope technique (Zilversmit 1972; Cayen and Dvornik 1979) for determining cholesterol absorption in rats.

PROCEDURE

Male Sprague-Dawley rats weighing 200–225 g are fed with a diet containing 5.5% peanut oil, 0.5% cholic acid and 1.5% cholesterol with or without (controls) drugs for 1 week. On the last day, food is removed at 8:00 A.M. and the isotopes are administered beginning at 2:00 P.M. [^{3}H]cholesterol (13 μCi/rat) is given by oral gavage and [^{14}C]cholesterol (1.5 μCi/rat) is given is given by tail vein injection. The [^{3}H]cholesterol is prepared as an emulsion by dissolving 125 mg cholesterol in 1 625 mg olive oil. The oil phase is suspended by sonication in 25 ml of water containing 156 mg taurocholate (sodium salt). Each animal receives 1 ml. The intravenous dose is prepared by drying the labeled cholesterol (50 μCi), and then adding 300 μl warm ethanol followed by 12.5 ml of saline. Each animal receives 0.5 ml of this colloidal suspension. The rats are allowed to consume their respective diets at 3:00 P.M., and are sacrificed 48 h after the isotope administration.

EVALUATION

The percentage of an oral dose of cholesterol absorbed is calculated from the plasma isotope ratio (% of the oral dose in 2 ml plasma/of the intravenous dose in 2 ml plasma × 100).

FURTHER IN VIVO METHODS

Heider et al. (1983) measured cholesterol absorption via the Zilversmit dual isotope method in rabbits.

Gillies et al. (1990) studied the regulation of ACAT activity by a cholesterol substrate pool during the progression and regression phases of atherosclerosis in rabbits with dietary induced atherosclerosis.

Krause et al. (1993b) compared the activities of two ACAT-inhibitors in normocholesterolemic and hypercholesterolemic rats, rabbits, guinea pigs and dogs.

Nagata et al. (1995) tested a new ACAT inhibitor in diet-induced atherosclerosis formation in female C57BL/6J mice.

Bocan et al. (1993) described a reduction of VLDL and vessel wall cholesteryl ester content in Yucatan micropigs after treatment with an ACAT inhibitor.

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M.4.1.4 Lymph fistula model for cholesterol absorption

PURPOSE AND RATIONALE

Direct evidence for an inhibitory effect on cholesterol absorption can be obtained by the lymph-fistula model in rats. This model also provides an indication as to the duration of inhibition and the relative selectivity of the compound on the absorption of cholesterol versus triglyceride and phospholipid.

PROCEDURE

Rats are anesthetized by an intramuscular injection of tiletamine/zolazepam (Telazol, 40 mg/kg). Silicon rubber cannulae are placed into the main mesenteric lymph duct and into the duodenum and secured with sutures. Animals are allowed to recover from surgery overnight in restraining cages while infused intraduodenally with 2% dextrose in saline containing 0.03% KCl (2.5 ml/h). Drinking water is allowed at libitum during this recovery period.

At 6:00 A.M. the following day, the drinking water is removed and a 2-h basal lymph sample is collected. Then, the animals are given the ACAT inhibitor at a specified dose as a single bolus into the duodenal cannula using a aqueous CMC/Tween suspension vehicle. Controls receive a bolus injection of the vehicle alone. Immediately after the drug dose, a lipid emulsion containing 0.1% cholesterol, 0.11% sodium taurocholate, 15% Intralipid (20%, Kabivitrium Inc.), 2.4% safflower oil, and 82.6% saline is infused into the duodenal cannula (3 ml/h). Then, four 2-h lymph collections are obtained. The lymph samples are extracted into hexane in the presence of a stigmasterol internal standard. Total and free cholesterol are quantitated by liquid gas chromatography.

EVALUATION

Esterified cholesterol of lymph is determined from difference between total and free cholesterol.

MODIFICATIONS OF THE METHOD

The lymph fistula model can also be used to examine the effect of ACAT inhibitors on the absorption of endogenous (i.e. biliary) cholesterol. Cannulated rats are infused intraduodenally with the saline/dextrose solution to which 2% whole rat bile is added containing [^{14}C]cholesterol. No nonradiolabeled lipid other than that in bile is infused into the animals, and hence, lymph cholesterol is exclusively of biliary origin. Hourly collections of lymph are obtained with the use of fraction collectors. Lymph is extracted with 3 volumes of ethylacetate-acetone 2 : 1 (Slayback et al. 1977). The total ^{14}C label of an aliquot of lymph extract is determined by liquid scintillation spectroscopy. The lymph extracts are resuspended in 100 µl chloroform-methanol 2 : 1 and spotted on Whatman LK6D TCL plates. The plates are developed in hexane-diethylether-acetic acid 85 : 15 : 1. The distribution of ^{14}C label between the cholesteryl ester and cholesterol bands are visualized and quantified by exposing the plates to phosphor imaging plates for 16 h and then scanning the imaging screens on a Molecular Dynamics Phosphorimager.

Clark and Tercyak (1984) studied the absorption of cholesterol during inhibition of mucosal acyl-CoA: cholesterol acyltransferase in mesenteric lymph fistula rats with normal pancreatic function.

Åkerlund and Björkhem (1990), Björkhem et al. (1993) used the lymph-fistula model in rats for studies on the link between HMG-CoA reductase and cholesterol 7α-hydroxylase. The thoracic lymph duct was cannulated just proximal to the cisterna magna through an abdominal approach. The proximal part of the lymph duct was ligated. The cannula exited at the back of the animal. The lymph was allowed to flow freely from the animal to the bottom of the metabolic cages. Under these conditions, the rats could move freely in the cages during the lymphatic drainage.

CRITICAL ASSESSMENT OF THE METHOD

Gallo et al. (1987) found normal cholesterol absorption in rats in which intestinal acyl co-enzyme A:cholesterol acyltransferase activity was significantly reduced by ACAT-inhibitors. This challenges the value of ACAT-inhibition at least in normal individuals.

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M.5
Interruption of bile acid recirculation

M.5.0.1
Cholestyramine binding

PURPOSE AND RATIONALE

Cholesterol is metabolized in the liver by oxidation to bile acids which undergo enterohepatic circulation. In the untreated state, approximately 95% of the bile acids that are secreted are reabsorbed and returned to the liver, while the small loss is replaced by de novo biosynthesis from cholesterol. Increased excretion of bile acids with the feces increases the rate of oxidation of cholesterol in the liver leading to a partial depletion of the hepatic cholesterol pool. A compensatory increase in uptake via the LDL receptors results in lower serum LDL levels. This can be achieved by addition of a bile acid binding resin, e.g., cholestyramine, to the food. The binding of unconjugated and conjugated bile-salt anions can be tested *in vitro* (Johns and Bates 1969).

PROCEDURE

Rabbits weighing 2.5–3 kg are switched from standard food to a diet containing 10–20% polymeric basic-anion exchanging resin, e.g. cholestyramine. Cholesterol levels in serum are measured at the beginning and at the end of a 4 weeks feeding period.

EVALUATION

Cholesterol levels as means ±SD are calculated for controls and treated animals and compared by statistical analysis.

MODIFICATION OF THE METHOD

Tennent et al. (1960) tested polymeric organic bases for action on blood cholesterol in 4-day experiments and in experiments of 7–8 weeks duration in cholesterol-fed White Leghorn cockerels. The birds were given a diet containing 2% cholesterol and 5% cotton-seed oil with or without addition of polymeric bases. The increase of cholesterol and the incidence of aortic atheromatosis was decreased by polymeric organic bases.

Day (1990) compared the hypocholesterolemic activities of the bile acid sequestrants cholestyramine and cholestipol hydrochloride in cholesterol fed sea quail.

Quaternary ammonium conjugates of bile acid inhibited cholic acid binding and transport in everted ileal sacs of guinea pigs *in vitro* (Fears et al. 1990).

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M.6
Inhibition of lipid oxidation

M.6.0.1
General considerations

Oxidative modification of the low density lipoproteins (LDL) has been shown to cause accelerated degradation of LDL via the scavenger receptor pathway. Under conditions of high serum LDL levels, LDL particles can migrate into the subendothelial space where oxidation of LDL can occur. The actual oxidation process is believed to begin with lipid peroxidation, followed by fragmentation to result in short-chain aldehydes. These aldehydes can form adducts with the lysine residues of apo B, creating a new epitope which is recognized by the scavenger receptor of macrophages.

During the same process, lecithin is converted to lysolecithin, which is a selective chemotactic agent for monocytes. The monocytes adhere to the arterial wall and penetrate through to the subendothelium. Once there, the monocyte changes to a tissue macrophage which takes up the oxidized LDL via the scavenger receptor. The uptake of oxidized LDL continues until the macrophage is engorged with cholesteryl esters ultimately forming a foam cell. Groups of these foam cells constitute a fatty streak. By inhibiting the oxidation of LDL, it is hoped that the modification of apo B and the production of chemotactic lysolecithin can be prevented.

The family of receptors for mammalian low-density proteins has been reviewed by Hussain et al. (1999).

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M.6.0.2
Inhibition of lipid peroxidation of isolated plasma low density lipoproteins

PURPOSE AND RATIONALE

Hypercholesterolemic Watanabe rabbits are considered to be a suitable model to study the effect of antioxidants as anti-atherosclerotic agents (Carew et al. 1987; Kita et al. 1987; Steinberg et al. 1988; Dresel et al. 1990). Plasma of Watanabe heritable hyperlipidemic (WHHL) rabbits is used to test the inhibition of Cu^{2+}-induced lipid peroxidation of isolated low density lipoproteins (LDL).

PROCEDURE

Animals of a modified Watanabe heritable hyperlipidemic rabbit strain (Gallagher et al. 1988) are used. The animals are fed over a period of 12 weeks with Purina rabbit chow diet with or without 1% of test compound or standard (probucol). Plasma samples are collected in Na_2EDTA (0.1% final concentration). LDL are isolated from each rabbit plasma using a sequential ultracentrifugation technique at d = 1.019–1.063 g/ml (Mao et al. 1983). LDL are then dialyzed against phosphate buffered saline (PBS, 0.01 M sodium phosphate, 0.12 M NaCl, pH 7.4) at 4 °C for 24 h.

For determination of LDL lipid peroxidation induced by Cu^{2+}, 100 µg of each LDL sample is adjusted to a volume of 1.5 ml with distilled water. Lipid peroxidation is initiated by addition of $CuSO_4$ to a final concentration of 5 µM followed by an incubation at 37 °C for 3 h. The reaction is stopped by adding 100 µl of 50 mM Na_2EDTA. Fifty micrograms of LDL from the reaction mixture are added to 1.5 ml of 20% trichloroacetic acid and vortexed. Finally, 1.5 ml of 0.67% thiobarbituric acid (TBA) in 0.05 N NaOH is added and the mixture is incubated at 90 °C for 30 min. Samples are centrifuged at 1 500 rpm for 10 min. The absorbance of the supernatant fractions is determined at 532 nm to estimate the content of lipid peroxides

(thiobarbituric acid-reactive substances). A standard curve (0–5 nmol) of malondialdehyde is generated using malondialdehyde bis(dimethyl acetal) as reference to determine the lipid peroxidation content in Cu^{2+}-treated LDL.

EVALUATION

The content of lipid peroxide in LDL is plotted against the drug concentration in LDL fractions. The extent of Cu^{2+}-induced peroxidation decreases with increasing drug concentrations. The effects of test compounds are compared to the standard.

MODIFICATIONS OF THE METHOD

Inhibition of iron-dependent lipid peroxidation by test compounds was measured by Braughler et al. (1987), Yoshioka et al. 1989).

Yamamoto et al. (1986) studied the effects of probucol on lipid storage in macrophages *in vitro* in the presence of acetylated low density lipoprotein using macrophage-like cells (UE-12) established from a human histiocytic lymphoma cell line.

Barnhart et al. (1989) used LDL from human plasma to study the concentration-dependent anti-oxidant activity of probucol.

Parthasarathy et al. (1986) incubated LDL from human plasma samples with rabbit aortic endothelial cells and measured the increase in electrophoretic mobility, the increase in peroxides, and the increase in subsequent susceptibility to macrophage degradation.

Mansuy et al. (1986) studied the inhibition of lipid peroxidation induced in liver microsomes either chemically by $FeSO_4$ and reducing agents (cysteine or ascorbate) or enzymatically by NADPH and CCl_4.

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M.6.0.3 Internalization of labeled LDL into HepG2 cells

PURPOSE AND RATIONALE

Enhanced uptake of low density lipoproteins (LDL) via the LDL receptor into liver cells results in reduced plasma cholesterol levels. This can be tested in the cultured hepatoma cell line HepG2.

PROCEDURE

Heterozygous WHHL rabbits are treated with the test compound over a period of 4 weeks. Blood is withdrawn twice weekly before, during, and after treatment for determination of total plasma cholesterol, low density lipoprotein cholesterol, total triglycerides, and high density lipoprotein cholesterol. Lipoprotein-deficient serum is prepared by ultracentrifugation (Goldstein et al. 1983). Serum of treated and untreated animals is used for the uptake assay being prepared by centrifugation to remove the clots, followed by heat inactivation of the complement system and sterilization through a 0.45-µm filter. In each preparation, 2 mg LDL (*d*, 1.019 to 1.050 g/ml) at 10 mg/ml are iodinated by the monochloride method to a specific activity of 300 cpm/ng (Huettinger et al. 1984).

HepG2 cells are grown in 5 cm dishes in Eagle's minimum essential medium to 60% confluence. Serum prepared from treated animals and from the same animals in the pretreatment period is added from 1% to 50% to the medium used to grow HepG2 cells. After incubation of 18 h, cells are washed and incubated in Eagle's minimum essential medium plus 2% bovine serum albumin and 8 µg labeled LDL for 3 h. The cells are then washed and solubilized in sodium hydroxide, and the content of each dish is counted for radioactivity. An aliquot is used to determine protein content.

EVALUATION

Values are given for specific uptake, which is calculated from the difference of total uptake minus uptake measured when a 40-fold excess of unlabeled LDL is present in the incubation medium of duplicate or triplicate incubations. The effect of the test drug is demonstrated by an increase of uptake with increasing percentage of serum of treated animals containing the active drug.

MODIFICATIONS OF THE METHOD

Sprague et al. (1993) measured the inhibition of scavenger receptor-mediated modified low-density lipoprotein endocytosis in cultured bovine aortic endothelial cells.

Takano and Mowri (1990) produced a monoclonal antibody which recognizes peroxidized lipoproteins.

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Chapter N
Endocrinology[1]

[1] Review of this edition by J. Sandow.

N.1 Endocrine survey

PURPOSE AND RATIONALE

The effect of steroid and peptide hormones as well as the influence of drugs on the endocrine system are detected by repeated administration of the test substances. After administration during a period of 1–4 weeks, the endocrine glands and the organs depending on hormonal influences are weighed. Furthermore, the hormonal content of the endocrine glands and blood levels of hormones determined.

PROCEDURE

Separate groups of 5–10 male and female Sprague-Daw-ley rats weighing 55–65 g are used. For some compounds, groups of 200 or 300 g body weight may be required. They are treated daily over a period of 6–12 days with the test compound by the intended route of orally) or by subcutaneous injections. For toxicological studies, a treatment period of 4 weeks is preferable. A similar protocol is applied in chronic toxicity studies in rats and dogs. On the day after the last application, the animals are sacrificed, weighed, and the following parameters determined:

Parameter	Optional
Pituitary-adrenal axis	
Pituitary weight	Corticotropin-releasing hormone in hypothalamus
Adrenal weight	Pituitary content of adrenocorticotropin and vasopressin
Thymus weight	Adrenal content of corticosterone and aldosterone; Corticosterone and aldosterone blood level; Electrolyte (Na^+, K^+, Ca^{2+}, Cl^-) concentrations in serum; Urinary excretion of corticosterone and aldosterone
Pituitary-gonadal axis	
Male reproduction	
Pituitary weight	LH-RH in hypothalamus
Weight of testes	Pituitary content of FSH, LH and prolactin
Weight of seminal vesicles	Blood levels of FSH, LH and prolactin
Weight of ventral prostate	Testosterone content in testes and serum
Weight of musculus levator ani	Growth hormone in pituitary and serum
Female reproduction	
Pituitary weight	LH-RH in hypothalamus
Weight of ovaries	Pituitary content of FSH, LH and prolactin
Weight of uterus	Blood levels of FSH, LH and prolactin; Content of estradiol and progesterone in ovaries and serum
Pituitary-thyroid axis	
Pituitary weight	TRH in hypothalamus
Thyroid weight	Pituitary content of thyrotropin; Serum content of TSH, T3 and T4

After dissection, organ weights are recorded, serum or plasma and the endocrine glands are stored frozen at –20 °C for further determination of hormonal content. This method may include histology, *in situ* hybridization etc. as required.

EVALUATION

The mean value of each parameter of the treated groups are compared with the values of the vehicle control group.

MODIFICATIONS OF THE METHOD

Many modifications are possible, e.g., extension of the treatment period up to 4 weeks in order to detect toxicological effects after long-term administration, inclusion of supplementary parameters, satellite groups. Further specific tests are necessary to clarify the mode of action.

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N.2
Adrenal steroid hormones

N.2.0.1
Adrenalectomy in rats

PURPOSE AND RATIONALE

The classical evaluation of hormone functions is the surgical ablation of the hormone producing endocrine gland and the substitution with exogenously administered substances. Most studies on the physiological role of adrenocortical hormones and the pharmacological effects of corticosteroids were performed in adrenalectomized rats.

PROCEDURE

Sprague-Dawley or Wistar rats of either sex weighing 120–150 g are used. The dorsal fur is shaved, the rat is anesthetized with ether and placed on a block ($8 \times 3 \times 2$ cm) in order to elevate the viscera. A transverse incision about 5 mm long is made in the midline at the costovertebral angle. To remove the left adrenal gland, the skin is retracted to the ventral side and the lumbar muscles incised just superior and anterior to the splenic shadow. In this way, the adrenal gland appears directly beneath the incision and no exteriorization of the kidney is necessary. The periadrenal tissue is grasped between the kidney and the adrenal by small curved forceps and the intact gland together with the periadrenal fat and the mesenteric attachments is removed in toto. The adrenal gland and its capsule are not touched and any remnants of the capsule to which cortical tissue may adhere are removed. The bleeding is negligible in young animals so that no vessels need be tied off.

After ablation of the left adrenal, the animal is turned around and the right gland is removed through the original skin incision. A small incision through the lumbar muscles is made just above and anterior to the prominent lumbocostal artery which is seen near the costal margin. The curved forceps are inserted over the kidney and by elevating the liver, which covers the adrenal on this side, the gland is brought into view and grasped by the forceps removing again the intact gland with the periadrenal fat and the mesenteric attachments. The incisions made in the lumbar muscles need not exceed 3 mm in length and may be made by spreading the blades of a pair of scissors, hemostasis or closure by sutures is not necessary. The incision is closed by a skin clip. The entire procedure is done in a time sufficiently short to avoid long-acting anesthetics. The animals appear normal in every aspect within a few min following the operation.

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N.2.1
Glucocorticoid activity

N.2.1.1
In vitro methods for glucocorticoid hormones

N.2.1.1.1
Corticoid receptor binding

PURPOSE AND RATIONALE

Steroid hormone receptors are intracellular metalloproteins which bind steroids with high affinity and high specificity. The lipophilic steroids enter the cell by diffusion. The binding of the hormone to its specific receptors results in a series of conformational changes,

leading to an increase in affinity for specific DNA regulatory elements (steroid responsive elements). After binding, transcription of target genes is enhanced and mRNAs are produced. These mRNAs are then translocated to the cytoplasm and translated into proteins regulating cellular metabolism and initiating the cellular responses. The structure and functions of the steroid receptor superfamily have been reviewed by Carson-Jurica et al. (1990), Lazar (1991), Barnes and Adcock (1993), Distelhorst (1993), Power et al. (1993), Brinkmann (1994), Ojasoo et al. (1994, 1995), Wittliff and Raffelsberger (1995), Beato et al. (1996), Jensen (1996). The relative binding affinities for the glucocorticoid receptor of rat liver or thymus cytosol can be measured by competitive displacement of [^{3}H]-dexamethasone (Raynaud et al. 1979; Wojnar et al. 1986; Ueno et al. 1991).

PROCEDURE

The steroid receptor preparation is obtained from rats. Male Wistar rats weighing 140–150 g are adrenalectomized under anesthesia. Two days later, the liver is excised and homogenized in a 10 fold buffer containing 50 mM Tris-HCl, 1 mM EDTA, 2 mM dithiothreitol, 10 mM Na_2MO_4, and 10% glycerol (pH 7.4). The homogenate is centrifuged for 1 h at 105 000 *g* at 4 °C and collected as the supernatant fraction. The cytosol is mixed with 5 nM [^{3}H]-dexamethasone in the presence or absence of appropriate concentrations of competitors and incubated for 2 h at 4 °C. The reaction is terminated by the addition of hydroxyapatite in order to separate the receptor-steroid complex from the free [^{3}H]-dexamethasone. The radioactivity bound to the receptors is determined by liquid scintillation spectrometry.

EVALUATION

IC_{50} values are estimated by probit analysis.

MODIFICATIONS OF THE METHOD

Various modifications of the glucocorticoid receptor assay have been reported in the literature. Cytosols have been prepared from rat liver, from cultured rat hepatoma cells (Rousseau and Schmit 1977), from normal human lymphocytes (Steiner and Wittliff 1985), thymocytes from rat thymus gland (Lefebvre et al. 1988), human leukemic lymphoid cell line, CEM C7 (Srivastava and Thompson 1990), rat lung (Druzgala et al. 1991; Hochhaus et al. 1991), human lung (Rohdewald et al. 1985), rat hippocampus (Jacobson et al. 1993) and other sources.

Differentiation of type I (mineralocorticoid) and type II (glucocorticoid) receptors has been attempted (Spencer et al. 1990; Jacobson et al. 1993).

A new affinity label for glucocorticoid receptors was described by Lopez and Simons (1991).

The solution structure of the glucocorticoid receptor DNA-binding domain has been determined (Härd et al. 1990).

Berger et al. (1992) used transient co-transfection of receptor cDNA and suitable reporter genes to study human glucocorticoid receptor function in a CV-1 mammalian cell line. A variety of natural and synthetic steroids were analyzed for their ability to activate gene expression through the human glucocorticoid receptor and to bind to extracts of cells expressing the human glucocorticoid receptor cDNA. A good correlation between both *in vitro* parameters and *in vivo* anti-inflammatory activity was reported for most of the tested steroids.

Guo et al. (1995) tested the binding of various steroids to the synaptic plasma membrane which may be a novel type of glucocorticoid receptor on neuronal membrane being significantly different from the cytosolic glucocorticoid receptors.

A general survey on steroid hormones, receptors and antagonists was given by Jensen (1996).

Teutsch et al. (1995) discussed structure-activity correlation of hormone antagonists ("antihormones").

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N.2.1.1.2 Transactivation assay for glucocorticoids

PURPOSE AND RATIONALE

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor interacts with hormone responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon (1988). The hormone-dependent transcriptional activation can be determined in tissue culture by transfection of the steroid receptor under investigation and a reporter gene linked to a hormonally responsive promotor into cells. The transactivation assay allows determination of the agonistic and also the antagonistic potency of a given compound, by induction or inhibition of reporter gene activity (Fuhrmann et al. 1992).

PROCEDURE

Vector construct

The expression plasmid pHGO containing the full-length coding sequence of the human glucocorticoid receptor driven from the SV 40 early promotor is prepared according to Hollenberg et al. (1985). The pMMTV-CAT plasmid containing the mouse mammary tumor virus promotor linked to a chloramphenicol acetyltransferase gene is prepared according to Cato et al. (1986).

Cell culture and transfections

CV-1 cells and COS-1 cells for transient infections are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 4 mmol/L L-glutamine, penicillin and streptomycin. Stable and transient transfections are performed using Lipofectin reagent (Gibco BRL) according to a procedure recommended by Felgner and Holm (1989), Felgner et al. (1987). Stable transfections are carried out according to Fuhrmann et al. (1992). For transient

transfection, 1×10^6 COS-1 or CV-1 cells, respectively, are plated onto 100-mm dishes one day prior to transfection. Cells are typically about 80% confluent after 24 h. Before transfection, cells are washed twice with 1 ml Opti-MEM (Gibco BRL) per dish. For each dish, 5 µg pHGO (hGR expression plasmid) and 5 µg pMMTV-CAT are diluted with 1 ml Opti-MEM; in addition, 50 µg Lipofectin Reagent is diluted with 1 ml Opti-MEM. Next, DNA and Lipofectin Reagent dilutions are combined in a polystyrene snap-cup tube to obtain 2 ml of transfection solution per dish, gently mixed, incubated at room temperature for 5 min, and added to the washed cells. After 5 h the transfection solution is replaced by 6 ml DMEM supplemented with 10% fetal calf serum.

To study the effect of the test hormones, transiently transfected cells are trypsinized, pooled and replated onto 60-mm dishes at a density of 4.5×10^5 per dish 24 h after transfection. Stably transfected cells are seeded onto 6-well dishes (1×10^5 cells per dish). Cells are cultured in medium supplemented with 3% charcoal-stripped fetal calf serum and the appropriate hormones for 48 h. As negative control for the reporter gene induction, cells are cultured with 1% ethanol. Transactivation assays with transiently or stably transfected cells are carried out at least three times.

CAT assay

Transiently transfected cells and stably transfected cells are disrupted by freezing (ethanol/dry ice bath) and thawing (37 °C water bath) three times. Protein concentrations of the cell extract are determined according to Bradford (1976). The CAT assay is performed according to Gorman et al. (1982). After the cells are spun for 15 min in an Eppendorf microfuge at 4 °C, the supernatants are removed and assayed for enzyme activity. The assay mixture contains (in a final volume of 180 µl) 100 µl of 0.25 M Tris-hydrochloride (pH 7.5), 20 µl of cell extract, 1 µCi of [^{14}C]chloramphenicol (50 µCi/mmol; New England Nuclear Corp.), and 20 µl of 4 mM acetyl coenzyme A. Controls contain CAT (0.01 U; P.L. Biochemicals Inc.) instead of cell extract. All of the reagents except coenzyme A are preincubated together for 5 min at 37 °C. After equilibration is reached at this temperature, the reaction is started by adding coenzyme A. The reaction is stopped with 2 ml cold ethyl acetate, which is also used to extract the chloramphenicol. The organic layer is dried and taken up in 30 µl of ethyl acetate, spotted on silica gel thin-layer plates, and run with chloroform-methanol (95 : 5), ascending). After autoradiography of the separated acetyl chloramphenicol forms, the spots are cut out and counted. Data are expressed as the amount of chloramphenicol acetylated by 20 µl of extract.

EVALUATION

CAT activity is calculated as percent conversion from chloramphenicol to acetylated chloramphenicol. Concentration-response curves for CAT induction are established to determine the potency of the test hormone. Dexamethasone (10^{-10} to 10^{-6} mol/l) serves as standard.

For antiglucocorticoid activity, CAT activity in the presence of 10^{-8} mol/l dexamethasone is set as 100% and relative CAT activity is calculated as percentage of this value. Concentration-response curves for CAT inhibition are established with increasing concentrations of the antihormone RU 486as the standard inhibitor.

CRITICAL ASSESSMENT OF THE METHOD

Hollon and Yoshimura (1989) examined the causes for high variability in data from enzymatic transient gene expression assays. Their results strongly suggested that variation in transfection efficacy is the major cause of data variation and can seriously compromise valid interpretation of data.

MODIFICATIONS OF THE METHOD

White et al. (1994) described a simple and sensitive **high-throughput assay** for steroid agonists and antagonists. A DNA cassette, containing a synthetic steroid-inducible promotor controlling the expression of a bacterial chloramphenicol acetyltransferase gene (GRE5-CAT), was inserted into an Epstein-Barr virus episomal vector which replicates autonomously in primate and human cells. This promotor/reporter system was used to generate two stably transfected human cell lines. In the cervical carcinoma cell line HeLa, which expresses high level of glucocorticoid receptor, the GRE5 promotor is inducible over 100-fold by the synthetic glucocorticoid dexamethasone. In the breast carcinoma cell line T47D, which expresses progesterone and androgen receptors, the GRE5 promotor is inducible over 100-fold by either progesterone or dihydrotestosterone. These cell lines can be used to screen large numbers of natural and synthetic steroid agonists and antagonists in microtiter wells using a colorimetric chloramphenicol acetyltransferase assay.

Dias et al. (1998) recommended the use of Cre recombinase in mammalian cells for high through-put screening of chemical libraries to identify new receptor ligands. A translational fusion of Cre recombinase and the ligand binding domain of the human glucocorticoid receptor was transfected into mammalian cells with the *loxP*/luciferase reporter gene. A stable transfected clone was isolated and used to characterize the kinetics, ligand specificity, and dose response to various receptor ligands.

Vayssière et al. (1997) studied synthetic glucocorticoids that dissociate transactivation and AP-transrepression.

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N.2.1.1.3 Induction of tyrosine aminotransferase in hepatoma cells

PURPOSE AND RATIONALE

The synthesis of tyrosine aminotransferase in hepatoma tissue culture (HTC) cells (Thompson et al. 1966) can be induced by glucocorticoids. This effect can be abolished by glucocorticoid antagonists.

PROCEDURE

Hepatoma tissue culture (HTC) cells are grown in suspension to a density of about 8×18^5 cells/ml. The cells are washed 3 times at 0 °C in a total volume of buffered saline equivalent to half of the culture medium. The suspension is centrifuged and cell pellets resuspended in serum free medium containing 0.1% BSA and 0.1% $NaHCO_3$. Ethanol solutions of standard (dexamethasone) and test steroids in various concentrations are added to 10 ml aliquots of cell suspension, final ethanol concentration not to exceed 0.5%. After 16 h of incubation in tightly capped flasks on a rotary shaker (100 rev/min) at 37 °C, the cells are harvested and tyrosine aminotransferase determined. The cells are washed twice and then disrupted with an ultrasonicator. The enzyme is assayed at 37 °C by conversion of p-hydoxyphenylpyruvate to p-hydroxybenzaldehyde (Diamondstone 1966). One unit of activity represents the conversion of 1 µmole of p-hydroxy-phenylpyruvate/min.

EVALUATION

Enzyme specific activity is expressed in milliunits of tyrosine aminotransferase/mg of cell protein. Dose-response curves are established for the standard and the test preparation allowing the calculation of potency ratios with confidence limits.

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N.2.1.1.4 Effect on T-lymphocytes

PURPOSE AND RATIONALE

Corticosteroids as immunosuppressive agents influence T-lymphocyte function. Snijdewint et al. (1995) determined *in vitro* dose-dependent inhibition of Th1- and Th2-type cytokine production by various corticosteroids.

PROCEDURE

Culture media

T-lymphocyte clones are maintained and expanded in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% pooled c-inactivated human serum and gentamycin (80 µg/ml). Experiments are performed in IMDM supplemented with 10% FCS, human transferrin (35 µg/ml) 2-ME (3.5 µg/l) and human insulin (1.75 IE/ml).

Peripheral blood lymphocytes and T-lymphocyte clones

Peripheral blood mononuclear cells are prepared from heparinized venous blood of healthy volunteers by flotation on Lymphoprep (Nycomed, 1.077 g/ml) and the peripheral blood lymphocytes are separated by centrifugation on a discontinuous Percoll density gradient. Peripheral blood lymphocytes are collected form the interface between densities 1.977 g/ml and 1.061 g/ml, washed three times in HBSS plus 2% FCS and suspended in IMDM plus 10% FCS. Isolated peripheral blood lymphocytes (0.5×10^6 cells/ml) are stimulated with CD2 mAb plus CD28 mAb (1.1000 final dilution of ascites) in the absence or presence of corticosteroids (Van der Pouw-Kraan et al. 1992). Supernatants are collected after 48 h for IFN-γ and IL-2 measurement, and after 96 h for IL-4 measurement.

Housedust mite (*Dermatophagoides pteronyssinus*)-specific T-lymphocyte clones are generated from peripheral blood and skin of atopic individuals (Van der Heijden et al. 1991). These clones are Th0 typed, capable of producing both IFN-γ and IL-4 (Kapsenberg et al. 1988). T-lymphocyte clone cells (0.5×10^6 cells/ml) are stimulated with CD2 and CD28 mAb (1.1000) plus 1 ng/ml PMA to induce optimal levels of cytokine production. Corticosteroids are added at the start of the stimulation. 24 h after stimulation, supernatants are collected and stored at –20 °C until cytokine production is analyzed.

Proliferation assay and cytokine measurements

Proliferation by peripheral blood lymphocytes is measured 48 or 96 h after stimulation in 96-well flat-bottom culture plates using 10^4 cells/well, the last 16 h in the presence of 13 kBq (0.33 µC)/well of [^{3}H]TdR (Radiochemical Centre, Amersham, UK). T-lymphocyte clones proliferation (10^4 cells/well) is similarly measured after stimulation for 40 h, again the last 16 h in the presence of [^{3}H]TdR. Incorporation of [^{3}H]TdR is determined by liquid scintillation spectroscopy.

Measurement of IFN-γ and IL-4 in the peripheral blood lymphocytes and T lymphocyte clones supernatants is performed with specific solid-phase sandwich ELISA systems (Van der Pouw-Kraan et al. 1992). For quantification of IL-2 production, a commercial ELISA kit is used.

IL-2R expression by T cells of peripheral blood lymphocytes is determined 48 h after stimulation by labelling with mouse anti-human CD25 mAb.

EVALUATION

Dose-response curves of inhibition of IFN-γ, IL-4 and IL-2 production as % of initial values in peripheral blood lymphocytes and in different T-lymphocyte clones are established for various corticosteroids in the range of 10^{-10} to 10^{-6} M.

All measurements are performed in duplicate of triplicate. Data are expressed as mean ±SEM and are evaluated statistically using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Mollison et al. (1999) compared a macrolactam inhibitor of T helper type 1 and T helper type 2 cytokine biosynthesis in various animal models with several steroid preparations, used for topical treatment of inflammatory skin diseases.

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N.2.1.1.5 Inhibition of cartilage degradation

PURPOSE AND RATIONALE

The efficacy of glucocorticosteroids after intra-articular injection has been clearly demonstrated in several animal models of osteoarthritis (Van den Berg et al. 1992; Pelletier et al. 1995). Augustine and Oleksyszyn (1997) used *in vitro* experiments to study the inhibition of degradation in bovine cartilage explants stimulated with concomitant plasminogen and interleukin-1α by various glucocorticosteroids.

PROCEDURE

Preparation of bovine articular cartilage explants

Bovine (calf) radiocarpal joints are acquired from a local abattoir immediately after sacrifice and transported on ice. The specimens are washed thoroughly and placed on ice containing 25% Povidine (10% Povidone-iodine topical solution). The specimens are then dissected in a sterile hood using good sterile technique. Media (DMEM containing 4.5 g/l D-glucose and L-glutamine, without sodium pyruvate) is supplemented with HEPES buffer(3.57 g/l) and sodium bicarbonate (3.7 g/l), and the pH adjusted to 7.4. The media is further supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 50 µg/ml L-ascorbic acid. The articulating cartilage surfaced are exposed, and the synovial fluid is wiped away with sterile gauze. A sterile cork-borer with a diameter of 3.5 mm is used to remove uniform cores of cartilage. The cores are placed in a sterile flask, washed for times with sterile media, and then placed in an incubator (37 °C, 5% CO_2/95% air, adequate humidity) and allowed to equilibrate for 1 h.

The cartilage disks are then labeled en mass with [^{35}S] sulfate at a concentration of 10 µC/ml for approximately 72–96 h, with hand-stirring every few hours. After labeling, explants are equilibrated with fresh media (minimum of 2 washes before use in experiments).

Inhibition of IL-1-induced cartilage degradation in bovine articular cartilage in the presence of human plasminogen

Individual explants are transferred to 96-well plates containing 250 µl of fresh media per well, with or without plasminogen and IL-1α, and with or without glucocorticosteroids. A negative control consists of media alone, while two positive controls are IL-1α alone, and plasminogen with IL-1α. All other groups contain the glucocorticosteroids along with recombinant human IL-1α (0.4 ng/ml) alone or concomitant human plasminogen (0.4 µM) plus IL-1α. Glucocorticosteroids are added in concentrations between 10 and 10 000 pM.

Control and experimental explants are incubated for approximately 96 h (four days) prior to counting a 50 µl sample of supernatant from each well. A 50 µl sample of a papain-digest of each explant is also counted.

EVALUATION

From the counts released into the supernatant over four days, and calculation of the total counts present (determined by digest counts), the data is expressed as % glycosaminoglycane release over the four days. The values for all groups with glucocorticosteroids present are then compared to the values for concomitant plasminogen and IL-1a without glucocorticosteroids, and percent inhibitions are calculated. Dose-response curves are established for each glucocorticosteroid.

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N.2.1.2 *In vivo* methods for glucocorticoid hormones

N.2.1.2.1 General considerations

Muscle work tests have been used in early stages of adrenal gland research (Bomskov 1937; Ingle 1944). The method is based upon the fact that muscular responsiveness is lost within a few hours following the removal of the adrenal glands and can be maintained by the administration of corticosteroids. Since the 11-oxygenated steroids are quite active and compounds such as desoxycorticosterone are practically without activity, it appears that the principal effect monitored is on carbohydrate metabolism. In view of more specific methods, the muscle work tests have only historical interest.

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N.2.1.2.2 Adrenal and thymus involution

PURPOSE AND RATIONALE
Repeated administration of corticosteroids causes both central and peripheral effects. In the immature rat, thymus involution is observed. The secretion of pituitary ACTH is blocked resulting in atrophy of the adrenal glands.

PROCEDURE
Groups of 6–10 immature male Sprague Dawley rats weighing 60–70 are injected subcutaneously daily for 6 days with 0.2 ml of a homogenized suspension of different doses of the test compound in 0.5% aqueous carboxymethylcellulose solution. The standard, hydrocortisone acetate is given in daily doses of 0.05 and 0.2 mg per animal. Controls receive the vehicle only. On the seventh day, the animals are sacrificed and the adrenal and thymus weights determined.

EVALUATION
The degree of involution of the thymus gland is a measure of the catabolic activity of the compound. Involution of the adrenals is a measure of the ability of the compound to inhibit the secretion of ACTH. Dose-response curves are established for both parameters and compared with hydrocortisone in order to calculate potency ratios.

MODIFICATIONS OF THE METHOD
The sensitivity of the test can be increased if the product of thymus and adrenal weight is calculated. Dose-response curves of the product are steeper than those of the individual glands (Laschet and Hohlweg 1960).

Inhibition of uridine incorporation into ribonucleic acid of thymocytes has been used as parameter for agonistic and antagonistic glucocorticoid activity (Gaignault et al. 1977).

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N.2.1.2.3 Eosinopenia test in adrenalectomized mice

PURPOSE AND RATIONALE
Glucocorticoids decrease the eosinophilic cell blood count in laboratory animals as well as in man. This effect was used to quantitate the potency of corticosteroids in adrenalectomized mice.

PROCEDURE
Male mice weighing 20–25 g (e.g., of Jax C57 Brown, subline cd strain) are adrenalectomized and maintained at 28 °C with 1% sodium chloride solution in water. Fifteen mg pellets of desoxycorticosterone acetate are implanted at the time of operation. Steroids are dissolved in benzyl alcohol and mixed with sesame oil (1 : 10). Three days after operation, the mice receive 5 µg epinephrine by subcutaneous injection and 4 h later 1–6 µg of hydrocortisone (or the test substance) in 0.03 ml oil. Three doses of the unknown substance and standard are tested, using 6 animals per dose. Blood samples are taken from the tail before and 3 h after steroid injection. Mice with fewer than 100 eosinophils per cubic millimeter of blood before injection are discarded.

EVALUATION
The percentage decrease of eosinophil blood count after 3 h is averaged per group. From dose-response curves, potency ratios are calculated.

CRITICAL ASSESSMENT OF THE METHOD
The test is relatively simple and has the advantage of requiring little material.

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N.2.1.2.4 Liver glycogen test in rats

PURPOSE AND RATIONALE

The liver glycogen deposition test, as described by Stafford et al. (1955) is a simple and specific test for glucocorticoid activity.

PROCEDURE

Male Sprague-Dawley rats weighing 140–160 g are adrenalectomized. They are fed stock laboratory diet and 1% sodium chloride solution. On the morning of the fourth postoperative day, food is withdrawn. On the morning of the fifth day, the drinking fluid is withdrawn and the rats are given the test compound by a single subcutaneous injection in appropriate dosages suspended in 0.5 ml sesame oil. Seven hours later, the rats are sacrificed. The livers are removed and blotted on filter paper to remove blood, weighed, dropped into flasks containing 10 ml hot 30% potassium hydroxide and digested on a hot plate. The digest is diluted to 100 ml and a 50-fold dilution of an aliquot is used for analysis.

Ten ml 0.2% anthrone in 95% sulfuric acid is slowly added to 5 ml of liver digest dilution with cooling. The mixture is heated in a boiling water bath for 10 min and then placed into cold water. Optical density is measured in a spectrophotometer at 620 µm using the anthrone-reagent as blank. Calibration curves are established using glucose as standard. Groups of 5 animals are used for each dose and for the vehicle controls. Three doses of test compound and of standard are used to find dose-response activities. Standard doses of hydrocortisone are 0.5, 1.0, and 2.0 mg per animal subcutaneously or 1.25, 2.5, and 5.0 mg per animal orally.

EVALUATION

The content of glycogen expressed as glucose is calculated per g liver weight. Dose-response curves are established for each compound and for the standard in order to calculate potency ratios.

MODIFICATIONS OF THE METHOD

The method can be used to determine the time course of activity and the duration of action of derivatives such as corticosteroid esters compared to the free alcohol (Vogel 1963, 1965) and for evaluation of topical and systemic activity (Alpermann et al. 1982).

The liver glycogen deposition in the **adrenalectomized mouse** has been used by Dorfman et al. (1946) and by Venning et al. (1946) to evaluate glucocorticoid activity.

Liver tryptophan peroxidase activity in the rat is decreased after adrenalectomy and increased after administration of corticosteroids (Knox and Auerbach 1955) indicating an early effect of corticoids.

The enzyme activity of tryptophane pyrrolase in **guinea pig liver** is increased by systemic administration of corticoids, but this effect is short-lived and achieved only with high s.c. doses (Albrecht et al. 1979). In contrast, the enzyme is markedly reduced by high-dosed and protracted epicutaneous application of dermatocorticoids.

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N.2.1.2.5 Anti-inflammatory tests

GENERAL CONSIDERATIONS

Most of the tests described in the Sect. H.3 (anti-inflammatory activity) have been used for evaluation of corticosteroids.

The measures of **acute inflammation**, such as

- **Ultraviolet erythema in guinea pigs** (see H.3.2.2.1),
- **Vascular permeability in rats** (see H.3.2.2.2), and
- **Paw edema in rats** (see H.3.2.2.6)

are less suitable, whereas the methods measuring **subacute inflammation** are very well suited, such as

- **Granuloma pouch test in rats** (see H.3.2.2.8),
- **Cotton granuloma test in rats** (see H.3.2.3.1),
- **Glass rod granuloma test in rats** (see H.3.2.3.3),
- **Sponge implantation technique** (see H.3.2.3.2).

The molecular mechanisms of the anti-inflammatory actions of glucocorticoids were reviewed by Barnes (1998). Development of dissociated steroids which are more active in transrepression (interaction with transcription factors) than transactivation (binding to glucocorticoid response elements) as reported by Vayssiere et al. (1997) was recommended.

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N.2.1.3 Influence of steroids on mechanical properties of connective tissue

N.2.1.3.1 Breaking strength of bones

PURPOSE AND RATIONALE

Patients with Cushing's disease or after long term treatment with corticosteroids have an increased susceptibility for bone fractures. The test was developed as a model for corticosteroid induced osteoporosis. Surprisingly, in chicken as well as in rats, low and medium doses of glucocorticosteroids increased breaking strength of bones dose-dependently whereas high, almost lethal doses induced a decrease of breaking strength.

PROCEDURE

White Leghorn chicken at an age of 14 days weighing 170 ±20 g are used. The animals are sacrificed after 14 days of treatment by intramuscular injections of doses from 0.2 up to 100 mg/kg cortisol (reference compound) or equivalent doses of glucocorticoids in oily solution. Body weight and comb weight are registered and the femur and tibia bones from both sites are freed from surrounding tissue and weighed. Length of bone (l) and outer diameter (D) of the diaphysis are measured using calipers. The bone is fastened on both ends, supported on edges 5 mm distant from the ends, and broken in the central portion of the diaphysis by a special apparatus assembled to an Instron®-instrument. Breaking load (P) is registered. The contralateral bone is cut by a small saw at the middle of the diaphyseal shaft into rings. Using a stereomicroscope, inner (d) and outer diameter (D) are measured in two perpendicular directions.

EVALUATION

Breaking strength (σ) of the hollow bones is calculated according to the technical formula for hollow cylinders:

$$\sigma = \frac{8}{\pi} \times \frac{P \times l \times D}{D^4 - d^4}$$

Doses between 0.2 and 2.0 mg/kg cortisol and 0.05 and 1.0 mg/kg prednisolone show a parallel increase of breaking strength allowing the calculation of potency ratios. With higher doses, the response changes to an inhibition of breaking strength. Excessive doses of 50 and 100 mg/kg cortisol which are in the range of the LD_{50} induce a decrease of breaking strength. It is concluded that the catabolic effect of glucocorticoids on bone matrix and mineral in this test can only be measured when using toxic doses.

MODIFICATIONS OF THE METHOD

In young and adult rats, 10 days of treatment with cortisol and other corticosteroids induce a dose-dependent increase of breaking strength, whereas with longer treatment (30 days) a bell-shaped dose-response (increase with low doses, decrease with high doses) is seen.

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N.2.1.3.2 Tensile strength of femoral epiphyseal cartilage in rats

PURPOSE AND RATIONALE

Epiphyseal cartilage of rats is very sensitive to treatment with hormones and other desmotropic drugs. Tensile strength of the distal femoral epiphyseal plate is decreased after adrenalectomy and restored or increased by treatment with low doses of corticosteroids.

PROCEDURE

Male Sprague-Dawley rats weighing 120 ±10 g are treated with several doses of corticosteroids or other hormones for periods between 1 and 14 days. After sacrifice, the hind legs are exarticulated in the hip joint and fastened at the collum femoris. Longitudinal tension always results in rupture of the distal femoral epiphyseal cartilage (Ther et al. 1963; Vogel and Ther 1964; Vogel 1969). The ultimate load of the femoral epiphyseal plate is registered by an Instron® instrument at an extension rate of 5 cm/min.

Single injections of doses between 10 and 100 mg/kg cortisol acetate or 2.5 and 40 mg/kg prednisolone acetate induce dose-dependent increase of tensile strength up to twice of the control levels after 24–48 h which subsides after 96 h. Repeated administration up to 15 days also results in a dose-dependent increase. However after administration of subtoxic doses for more than 2 months, the tensile strength falls below the age-matched control values. There is a maturation-dependent increase of tensile strength in untreated rats.

The effect of other hormones, e.g. gonadal steroids, on cartilage is much smaller than that of glucocorticosteroids.

EVALUATION

Dose-response curves for steroids can be established allowing the calculation of potency ratios in experiments with single dose or repeated dose administration.

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N.2.1.3.3 Tensile strength of tail tendons in rats

PURPOSE AND RATIONALE

The thread-like tail tendons of rats are very suitable for studying the mechanical properties of connective tissue. They are easy to prepare and consist predominantly of collagen. Like other organs of connective tissue, such as bone, cartilage, and skin, tensile strength of tail tendons is reduced after adrenalectomy and dose-dependently increased after short-term treatment with glucocorticosteroids.

PROCEDURE

Male Sprague-Dawley rats weighing 120 ±10 g are treated with increasing doses of corticosteroids or other hormones for periods between 1 and 14 days. After sacrifice, the tail is amputated at its basis. The tail skin and the last few coccygeal vertebrae are removed. Single tendons are pulled out from the dorsal and ventral bundles and kept in saline solution. Tendons of the same diameter (0.25 mm) are selected with a stereomicroscope. Since the diameter of the tendons may be influenced by long-term treatment of the animals, tendons of the same vertebral insertion are tested, alternatively. For this purpose, the 10^{th} vertebra is counted from the tail tip. All 6 tendons (4 on the ventral and 2 on the dorsal side) inserting on this vertebra are removed and tested. The tendons are fixed in special clamps at a distance of 2.0 cm and immersed in physiological saline. Stress-strain curves and ultimate loads are determined with an Instron®-instrument with an extension rate of 5 cm/min.

A single injection of cortisol acetate in doses from 10 to 100 mg/kg or prednisolone acetate in doses between 2 and 20 mg/kg prednisolone acetate causes a dose-dependent increase of tensile strength allowing the calculation of a potency ratio of 1 : 4 for prednisolone versus cortisol. Repeated injections of cortisol acetate in doses between 1.0 and 50 mg/kg sc or prednisolone acetate in doses between 0.05 and 50 mg/kg sc show the same dose-dependence and potency ratio. Tensile strength of tail tendons is similarly changed by treatment with gonadal steroids, depending on dose and duration of treatment, but to a much lesser degree than by corticosteroids.

EVALUATION

The potency ratios of glucocorticoids versus cortisol as the standard are calculated.

MODIFICATIONS OF THE METHOD

In addition to tensile strength, several other biophysical parameters can be determined in tail tendons, such

as hysteresis behavior (Vogel 1984). The ratio of energy input versus energy dissipation is calculated. Furthermore, dependence on strain rate and relaxation behavior under the influence of corticosteroids (Vogel 1989) as well as retardation (strain rate under constant load) has been studied (Vogel and Schorning 1990).

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N.2.1.3.4 Tensile strength of skin strips in rats

PURPOSE AND RATIONALE

Clinical experience shows unequivocally a decrease of skin thickness after long-term systemic or local treatment with corticosteroids. The mechanical properties of human skin after treatment with corticosteroids, however, have not been measured. Like with other connective tissues, in animal experiments a clear increase of tensile strength of skin has been found after short term administration of corticosteroids.

PROCEDURE

Male Sprague-Dawley rats with an initial weight of 120 ±10 g are treated with increasing doses of corticosteroids or other substances for periods between 1 and 14 days. After sacrifice, the back of the animals is shaved and a flap of skin measuring about 5×5 cm is removed. The skin flap is placed between 2 pieces of plastic material with known thickness. By these means, the actual thickness of the excised skin is measured by calipers. Two dumb-bell shaped specimens are cut with a special punch in perpendicular direction to the body axis having in the middle a width of 4 mm. They are fixed between the clamps of an Instron® instrument. In the usual experiments, stress-strain curves and ultimate loads are registered at a strain rate of 5 cm/min. From stress-strain curves, the values of ultimate load and ultimate extension are registered. The stress-strain curves show an almost linear part allowing the calculation of modulus of elasticity. Tensile strength is calculated from ultimate load divided by original cross-sectional area.

Dose response curves between 10 and 100 mg/kg cortisol acetate or 2 and 20 mg/kg prednisolone acetate show a sharp increase of ultimate load and tensile strength until 5 days, a continuous decrease of skin thickness upon longer treatment, and a decrease of ultimate load up to 2 months, which is below controls for ultimate load, but not for tensile strength (Vogel 1970a).

EVALUATION

Dose-response curves are established for various corticosteroids versus the standard cortisol allowing the calculation of potency ratios with confidence limits.

For a more detailed description of this method see Sect. P.11.2.1.1.

MODIFICATIONS OF THE METHOD

With a similar method, tensile strength of skin wounds after treatment with corticosteroids was studied in rats (Vogel 1970a,b). See also Sect. P.11.4.

Strain rate influences the values of ultimate load, tensile strength and modulus of elasticity, but not the effect of corticosteroids (Vogel 1972).

In contrast to parameters indicating strength and elasticity of collagen, the parameters indicating plasticity, like relaxation, hysteresis, strain at constant load (creep experiments), and isorheological behavior, are less dramatically changed by corticosteroids but indicate a decrease of viscosity or an increase of stiffness.

For a more detailed description of **relaxation experiments** (Vogel 1973) see Sect. P.11.2.1.5,

of **hysteresis experiments** (Vogel 1976, 1989) see Sect. P.11.2.1.6,

of **creep experiments** (strain at constant load) (Vogel 1977, 1989) see Sect. P.11.2.1.8,

of measurement of **isorheological behavior** (Vogel 1987, 1989) see Sect. P.11.2.1.7,

of ***in vivo* experiments** in rat skin (Vogel and Denkel 1985; Vogel 1989, 1993a). see Sect. P.11.3.

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N.2.1.4 Topical activity of glucocorticosteroids

N.2.1.4.1 Skin thickness and tensile strength

PURPOSE AND RATIONALE

From clinical experience it is known that a decrease of skin thickness occurs after long-term local treatment with corticosteroids. The mechanical properties of human skin after treatment with corticosteroids, however, have not been sufficiently measured. A clear increase of tensile strength of skin after short term local administration of corticosteroids has been found in animal experiments, whereas skin thickness decreased.

PROCEDURE

Male Sprague-Dawley rats with an initial weight of 150 ±10 g are used. The animals are shaved before and once again during treatment. The animals are treated once daily during 10 days with various concentrations of the test compound or the standard or with fixed concentrations of the test compound in different galenical preparations. The test material is applied in a volume of 0.2 ml to an area of 4 cm^2 to the shaven back skin. The animals are kept in individual cages to avoid systemic absorption by mutual licking. Two days after the last treatment the animals are sacrificed, a flap of skin measuring about 5×5 cm is removed and skin thickness is determined. The skin flap is placed between 2 pieces of plastic material with known thickness. By these means, the actual thickness of the excised skin is measured by calipers. Perpendicular to the body axis 2 dumb-bell shaped specimens are punched out. Stress-strain curves are recorded using an Instron® instrument. The following parameters are determined:

- load at rupture (ultimate load)
- extension at rupture (ultimate strain)
- skin thickness
- tensile strength (ultimate load divided by cross sectional area)
- modulus of elasticity (calculated from the straight part of the stress-strain curve).

EVALUATION

Dose-response curves are established for the parameters ultimate load, ultimate strain, skin thickness, tensile strength, and modulus of elasticity for the standard (prednisolone acetate) and the test compound allowing the calculation of potency ratios with confidence limits.

CRITICAL ASSESSMENT OF THE METHOD

In addition to corticosteroid-induced atrophy of the skin after topical application, mechanical parameters can be determined by this assay.

MODIFICATIONS OF THE METHOD

Kapp et al. (1977) used a special devised apparatus for **rats** covering the site of substance application, which guarantees an exclusive dermal absorption and excludes an oral ingestion of the steroid.

In addition to skin thickness and breaking strength, Töpert et al. (1990) determined thymus weight, water and glycosaminoglycan content in skin of rats after topical application of steroids over 30 days.

Iwasaki et al. (1995) measured the skin atrophy in Wistar rats by locally applied clobetasol-17-propionate, a synthetic glucocorticoid, and the influence of

simultaneously applied RU 486. Twenty-four h after the last application, the thickness of the skin was measured with a dial skin thickness gauge, with an accuracy of 0.01 mm. The treated skin was pinched lengthwise, and both skin surfaces were held vertically between two plastic discs of the gauge.

Hartop et al. (1978) measured transepithelial water loss (TEWL) in the skin of **essential fatty acid-deficient rats** (Prottey et al. 1976; Lowe and Stoughton 1977) after local treatment with corticosteroids.

Woodbury and Kligman (1992) recommended the **hairless mouse** as model for assaying the atrophogenicity of topical steroids. Epidermal atrophy was determined by the number of cell layers of viable epidermis on 5 fields under 400× magnification. Dermal thickness was determined under 400× magnification. The total number of sebocytes in all visualizable sebaceous glands was counted in 10 fields at 250× magnification.

Van den Hoven et al. (1991) used the hairless mouse as a model to distinguish between local and systemic atrophogenic effects of topical steroids. Male hairless mice (strain h/h-NMRI) were treated on the left flank with test preparations once daily for 3 weeks. During this time, body weight was measured and skinfold thickness of treated and untreated sides was determined using a graduated micrometer. On day 21, the animals were sacrificed and dermal thymidine uptake and weights of the thymus were determined.

Corticosteroid-induced skin atrophy in hairless mice can be prevented by tretinoin (Lesnik et al. 1989; Schwartz et al. 1994). In these studies, albino Skh-hairless-1 mice were treated topically twice daily with corticosteroids or the combination with tretinoin. At the end of the treatment, all mice were sacrificed and specimens of the dorsal skin were frozen for light microscopy and quantification of glycosaminoglycans, fibronectin, and collagen.

Wrench (1980) applied commercially available topical corticosteroid preparations to the proximal halves of albino mouse tail for 21 days and measured epidermal thickness by histology. All steroids caused epidermal thinning, except clobetasone butyrate.

Altmeyer and Buhl (1981) tested the antiacanthogenic effect of topically applied steroids after long-term treatment of **guinea pigs** (up to 118 days) by histological measurement of epithelial thickness. For delimitation of the measuring lines, the upper boundary was taken as that between the stratum granulosum and the stratum corneum, and the lower boundary as that between the basal cell layer and the corium.

Kajita et al. (1986) tested epidermal beta-adrenergic adenylate cyclase responses in **pigs** after topical application of glucocorticoids. A significant increase of this receptor response was observed 24 h following topical application of potent glucocorticoid ointments. Domestic white-haired pigs were anesthetized with 30 mg/kg Nembutal i.p. Four 5 × 5 cm areas were chosen and the following treatments were administered to each area: (1) topical administration of glucocorticoid ointments; (2) UVB irradiation alone (230 mJ/cm^2); (3) topical application of glucocorticoid ointments following UVB irradiation; (4) no treatment as a control. After 24 h the treated pigs were anesthetized again and skin specimens were obtained from the 4 areas by means of a Castroviejo keratome (Storz Instrument Co., St. Louis, Missouri) adjusted to 0.3 mm setting. Each skin slice obtained by the Castroviejo keratome was cut into 5 × 5 cm squares, which were washed 3 times in RPMI 1 640 medium and preincubated in the RPMI 1 640 medium for 37 °C to standardize the cyclic AMP level (Yoshikawa et al. 1975). After the preincubation, 2 pieces of skin squares were randomly selected and were incubated with various adenylate cyclase stimulators (Iizuka et al. 1985). The concentrations of epinephrine, adenosine, and histamine added to the incubation medium were 50 μM, 2 mM and 1 mM, respectively. The cyclic AMP content in skin squares was measured by radioimmunoassay using a Yamasa cyclic AMP assay kit (Yamasa Shoyu Co., Tokyo, Japan) after partial purification (Yoshikawa et al. 1975). The cyclic AMP phosphodiesterase activities in skin squares were measured by the method of Adachi et al. (1976) who purified multiple forms of pig epidermal cyclic nucleotide phosphodiesterases by DEAE-cellulose column chromatography.

In vitro experiments measuring the effects of retinoids and glucocorticoids on the beta-adrenergic adenylate cyclase system of pig skin epidermis were performed by Iizuka et al. (1985).

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N.2.1.4.2 Assay of topical glucocorticoid activity in transgenic mice

PURPOSE AND RATIONALE

Katchman et al. (1995) proposed a transgenic mouse model as biological assay of topical glucocorticosteroid potency.

PROCEDURE

A homozygous line of transgenic mice expressing 5.2 kilobases (kb) of the human elastin promoter region linked to the chloramphenicol acetyltransferase (CAT) reporter gene was developed (Hsu-Wong et al. 1994). For this purpose, 5.2 kb of human elastin 5'-flanking DNA is linked to a 0.7-kb CAT gene, followed by 0.3 kb of DNA with a polyadenylation signal. This linearized construct is injected into fertilized oocytes, and a line of transgenic mice expressing the human elastin promotor, as detected by CAT activity, is developed. These transgenic mice have no clinical phenotype and they do not express human elastin protein, as no part of the coding sequence is contained within the transgene. The human elastin promotor/CAT construct is expressed in a tissue-specific and developmentally regulated manner.

Four- or 5-day-old hairless pups, homozygous for the transgene, are used as test animals. In each experiment, pups of the same litter are used for comparison between glucocorticosteroid and control preparation in parallel. Steroid preparations are tested by applying 0.03 g uniformly on their dorsal surface (≈14 mg/cm^2). Control animals receive the same amount of creme base. The test animals are separated from each other and from their mothers and are sacrificed at different time points. Skin biopsies are removed immediately from the treated area.

For the CAT assay, skin is homogenized with a Polytron tissue homogenizer in 0.25 M TRIS hydrochloride (pH 7.5). The homogenates are then freeze-thawed three times and centrifuged at 10 000 *g* for 15 min. The protein content of the supernatant is determined by a commercial kit and aliquots, containing 100 mg of protein, are assayed for CAT activity in the linear range of the assay (Gorman et al. 1982) (see N.2.1.1.2).

EVALUATION

The significance of differences between different treatment groups is evaluated by Student's *t*-test.

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N.2.1.4.3 Effect on epidermal DNA synthesis

PURPOSE AND RATIONALE

Du Vivier et al. (1978), Marshall and Du Vivier (1978), Marshall et al. (1981), Clement et al. (1983) evaluated the local and systemic effects of topically applied corticosteroids on epidermal DNA synthesis in hairless mice.

PROCEDURE

Hairless mice of either sex, 2–4 months old, are dosed in groups of six with test preparations or control base. 0.03 ml of test drug formulation is spread over 3 cm^2 of the anterior dorsal skin. The posterior half of the dorsal surface of the animal is left untreated. Animals are injected subcutaneously with 25 mCi (methyl-^{3}H) thymidine, specific activity 5 Ci/mM in the right thigh 1 h before being sacrificed 6 or 24 h after application of test formulation.

The neck and back skin samples, with at least 2 cm unsampled skin left between them (to avoid inclusion of treated skin in the back sample) are removed. The epidermis is separated from the dermis by placing the sample, dermis downwards, on a stain-less steel plate at 57 °C for 25 s. The epidermis can then easily be separated from the dermis with a scalpel blade. The epidermal samples are wrapped in aluminum foil and stored at –20 °C until analysis.

Approximately 30 mg of epidermis in 5 ml of a 0.24 M sodium phosphate buffer solution, containing 8 M urea, 1% sodium lauryl sulfate and 1 mM EDTA at pH 6.8, is lysed using an ultrasonic disintegrator. DNA is extracted from the cell lysate by column chromatography using hydroxylapatite. The epidermal cell lysate is added to the hydroxylapatite column and washed with a buffer containing 0.24 M sodium phosphate and 8 M urea at pH 6.8 to remove RNA and protein. The urea is then removed with 50 ml of 0.14 M sodium phosphate buffer at pH 6.8. Double stranded DNA, which binds to hydroxylapatite under low salt buffer conditions, is eluted with 0.48 M sodium phosphate buffer at pH 6.8, and a 2 ml sample is collected.

The DNA concentration in the sample is determined by UV absorption at 260 nm in a spectrophotometer. 0.5 ml aliquots of the sample are added to 12 ml scintillation cocktail and 0.2 ml N HCl in scintillation vials. Each sample is prepared in triplicate and counted for 20 min/vial in a scintillation counter.

EVALUATION

The results are initially expressed as mean counts per minute (c.p.m.) for the vial triplets. The efficacy of counting for each sample is determined from the relevant channels ratio for that sample using a counting efficacy/channels ratio (quench correction) curve for this counting system. Mean c.p.m. are thus converted to mean disintegrations per minute (d.p.m.) and the results expressed as d.p.m./μg DNA. The significance of differences between different treatment groups is evaluated by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Marks et al. (1973) described a method for the assay of topical corticosteroids in mice. Sticky tape was applied five or six times to the dorsal skin of mature male hairless mice of the Harwell strain to remove the horny layer. A similar quantity of topical test preparations (about 70 mg) was applied on the stripped dorsal skin of each mouse. A dressing of cotton gauze lined with a plastic film was applied and secured with strapping staying for periods from 5 to 156 h. Four h before sacrifice the mice were intraperitoneally injected with 0.15 ml of a 0.1% colcemid solution together with 30 μCi of tritiated thymidine. After sacrifice the dorsal skin to which the preparation had been applied, was removed, fixed in 10% buffered formalin and stained with haematoxylin and eosin. For estimation of the mitotic index, cells in the prophase, anaphase, and metaphase stages were counted. To quantitate the labelling index, the number of labelled basal and suprabasal cells was estimated as a proportion of the total number of basal cells.

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Marshall RC, Du Vivier A (1978) Effect on epidermal DNA synthesis of the butyrate esters of clobetasone and clobetasol, and the propionate ester of clobetasol. Br J Dermatol 98:355–359

Marshall RC, Burrows M, Brookes LG, du Vivier A (1981) The effect of topical and systemic glucocorticosteroids on DNA synthesis in different tissues of the hairless mouse. Br J Dermatol 105:517–520

N.2.1.4.4 Induction of drug metabolizing enzymes

PURPOSE AND RATIONALE

Finnen et al. (1984, 1985) evaluated the effects of topical application of glucocorticosteroids on the

activity of drug metabolizing enzymes in the skin of adult hairless mice. The ability of steroid preparations to induce enzyme activity was related to their clinical potency.

PROCEDURE

Adult male and female hairless mice are treated with 0.3 g of test creams or cream base applied to the dorsal skin and rubbed in until no visible traces remain. Sixteen to 18 h after treatment, the animals are sacrificed, the treated area of skin removed, placed epidermis down on a curved surface and subcutaneous fat and muscle removed using a scalpel blade. Epidermis and dermis are separated by a heating technique (Thompson and Slaga 1976). The tissue is then placed in 0.1 M phosphate buffer pH 7.4 and minced finely using surgical scissors. Scissors minced tissue is then homogenized in ice-cold 0.1 M phosphate buffer pH 7.4 using a Polytron homogenizer. Whole skin homogenates are then centrifuged at 9 000 *g* for 20 min and the resulting supernatant used as source for the determination of ethoycoumarin O'dealkylase (Greenlee and Poland 1978). For the determination of ethoxyresorufin O'dealkylation (Pohl and Fouts 1980) the 9 000 *g* supernatants are further centrifuged at 100 000 *g* for 1 h, the resulting microsomal pellet resuspended in 0.1 M phosphate buffer pH 7.4, and used as the enzyme source. DNA content of the 9 000 *g* pellet is estimated by the diphenylamine reaction (Burton 1956).

EVALUATION

Results are shown as mean ±SE. The significance of differences between different treatment groups is evaluated by Student's *t*-test.

REFERENCES

Burton K (1956) A study on the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of desoxyribonucleic acid. Biochem J 62:401–437

Finnen MJ, Herdman ML, Shuster S (1984) Induction of drug metabolizing enzymes in the skin by topical steroids. J Steroid Biochem 20:1169–1173

Finnen MJ, Herdman ML, Shuster S (1985) Strain differences in the induction of mono-oxygenase activity in mouse skin by topical clobetasol propionate: evidence of a role for the HR locus. J Steroid Biochem 23:431–435

Greenlee WF, Poland A (1978) An improved assay of 7-ethoxycoumarin O'deethylase activity. Induction of hepatic enzyme activity in C5BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Pharmacol Exp Ther 205:596–606

Pohl RJ, Fouts JR (1980) A rapid method for assaying the metabolisms of 7-ethoxyresorufin by microsomal subcellular fractions. Analyt Biochem 107:150–155

Thompson S, Slaga TJ (1976) The effects of dexamethasone on mouse initiation skin and aryl hydrocarbon hydroxylase. Eur J Cancer 12:363–370

N.2.1.4.5 Cornea inflammation in rabbits

PURPOSE AND RATIONALE

Leibowitz et al. (1974, 1978, 1992), Cantrill et al. (1975) studied the anti-inflammatory efficacy of topical corticosteroids for treatment of glaucoma in rabbits.

PROCEDURE

New Zealand albino rabbits weighing 1.8–2.3 kg are anesthetized for approximately 5 min with 15 mg/kg i.v. thiamylal sodium. A corneal inflammatory response is produced by intralamellar inoculation of 0.03 ml of laboratory-grade clove oil. Before the induction of corneal inflammation, the rabbits are given three intravenous inoculations of 1.85×10^6 Bq/kg of an aqueous solution of tritiated thymidine (24.79×10^{10} Bq/mol) at 24-h intervals. The intracorneal injection of clove oil is given concomitantly with the third thymidine injection. Twenty-four hours later, therapy is initiated. A standard drop (0.05 ml) of drug is instilled hourly for a total of six doses, and then, after a lapse of 18 h, one drop is administered hourly for an additional total of seven doses.

Various corticosteroid preparations are tested in different concentrations. A control group is run with each experimental trial; control rabbits are handled in the same manner as experimental rabbits, except that the control rabbits receive either prednisolone acetate (positive control) or no treatment (negative control). Following completion of the treatment protocol, a 10-mm penetrating corneal button is removed by trephination, and tissue samples are placed in a commercially available solubilizing agent (Soluene 350, Packard Instrument Co.), 1 ml per cornea, until dissolved. The soluble samples are counted in a scintillation counter for a minimum of 10 min, quantitatively measuring the amount of radioactivity in each cornea.

EVALUATION

The significance of differences between different treatment groups is evaluated by Student's *t*-test.

REFERENCES

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Leibowitz HM, Kupferman A (1974) Anti-inflammatory effectiveness in the cornea of topically administered prednisolone. Invest Ophthalmol 13:757–763

Leibowitz HM, Kupferman A, Stewart HR, Kimbrough RL (1978) Evaluation of dexamethasone acetate as a topical ophthalmic formulation. Am J Ophthalmol 86:418–423

Leibowitz HM, Ryan WJ, Kupferman A (1992) Comparative anti-inflammatory efficacy of topical corticosteroids with low glaucoma-inducing potential. Arch Ophthalmol 110:118–120

N.2.1.4.6 Endotoxin-induced uveitis in rats

PURPOSE AND RATIONALE

When injected into the footpad of rats, *Salmonella* endotoxin, a lipopolysaccharide, produces uveitis. This is manifested in the anterior uvea and characterized by miosis with iris hyperemia, increase of protein content in aqueous humor and inflammatory cell accumulation in the anterior uvea and aqueous humor. Glucocorticoids strongly inhibits release of inflammatory mediators and this type of endotoxin-induced uveitis (Cousins et al. 1982; Tsuji et al. 1997).

PROCEDURE

Female inbred Lewis rats weighing about 160 g are used. Five hundred µg/kg Salmonella endotoxin dissolved in saline are injected into the footpads. Twelve hours later, the animals are sacrificed and both eyes of each animal used in the experiments. The anterior chamber of the eye is punctured, using a 27 gauge needle to collect the aqueous humor. Five-microliter aqueous humor samples are placed into 495 µl of phosphate-buffered saline containing 1% paraformalde-hyde. Cell number in the aqueous humor is then counted using a flow cytometry system. The cell number of both eyes of each animal is averaged for the statistical analysis of the results.

In topical applications, glucocorticoids are instilled (5 µl/eye) three times at 1 h before and 3 and 7 h after lipopolysaccharide injection. For systemic application, glucocorticoids are injected subcutaneously 3 h after lipopolysaccharide injection.

Total RNA is isolated from the iris-ciliary body with a mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). Each RNA sample is extracted from the pooled iris-ciliary bodies of both eyes of each animal. The extracted RNA is quantified after which 4 µg is used for DNA synthesis via polymerase chain reaction.

EVALUATION

Dunnetts's multiple comparison test is adopted for the statistical analysis of the results.

REFERENCES

Cousins SW, Rosenbaum JT, Guss RB, Egbert PR (1982) Ocular albumin fluorophotometric quantitation of endotoxin-induced vascular permeability. Infect Immun 36:730–736

Tsuji F, Sawa K, Kato M, Mibu H, Shirasawa E (1997) The effects of betamethasone derivatives on endotoxin-induced uveitis in rats. Exp Eye Res 64:31–36

Further methods measuring local or **topical activity** of corticosteroids described in Sect. H.3 **"Anti-inflammatory activity"**, such as

- **Oxazolone induced ear edema in mice** (see Sect. H.3.2.2.4),
- **Croton-oil induced ear edema in rats or mice** (see Sect. H.3.2.2.5),

are very suitable for evaluation of local corticosteroid activity

Furthermore, methods described in Chapter I **"Anti-arthrotic and immunmodulatory activity"**

- I.2.2.4 **Passive cutaneous anaphylaxis**
- I.2.2.6 **Delayed type hypersensitivity**

are recommended as models for testing topical corticosteroid potency.

N.2.1.5 Anti-glucocorticoid activity

N.2.1.5.1 Adrenal and thymus involution

PURPOSE AND RATIONALE

The involution of thymus and adrenal glands induced by hydrocortisone can be antagonized by compounds with antiglucocorticoid activity.

PROCEDURE

Groups of 6–10 immature male Sprague-Dawley rats weighing 60–70 are injected subcutaneously daily for 6 days with 0.2 ml of a suspension of different doses of the test compound in 0.5% aqueous carboxymethylcellulose solution, with hydrocortisone acetate as the agonist in daily doses of 0.2 mg per animal. Controls receive hydrocortisone acetate only. On the seventh day, the animals are sacrificed and the adrenal and thymus weights determined.

EVALUATION

The relative weight (quotient of adrenal or thymus weight (mg) and body weight (g)) is calculated for each rat. Means of the antagonist-treated groups are compared with the means of hydrocortisone only controls. Increase of thymus and adrenal weight relative to the hydrocortisone control indicates antiglucocorticoid activity.

MODIFICATIONS OF THE METHOD

Inhibition of tyrosine aminotransferase induced by corticosterone or dexamethasone was used by Vicent et al. (1997) to characterize the antiglucocorticoid properties of a novel synthetic steroid.

REFERENCES

Dorfman RI (1962) In: Dorfman RI (ed) Methods in Hormone Research, Vol II, Bioassay. Chapter 8, Corticoids, Academic Press, New York and London. pp 325–367

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N.2.2 Mineralocorticoid activity

N.2.2.1 *In vivo* methods

N.2.2.1.1 General considerations

Very early bioassays ("Survival tests") have been used to standardize extracts of adrenal cortex for mineralocorticoid activity. Several animal species do not survive after adrenalectomy. However, they can be kept alive by administration of mineralocorticoids. The first animal species used successfully was the adrenalectomized drake (Bülbring 1937). The methods employing adrenalectomized rats and mice (Dorfman 1962) suffer from the fact that in these species aberrant adrenal cortical tissue may occur, which is not removed by the classical adrenalectomy. In contrast, male Syrian golden hamsters weighing 50–60 g are very suitable to test adrenocortical activity (Junkmann 1955). For evaluation of mineralocorticoid activity these tests have been replaced by bioassays measuring electrolyte excretion in the urine.

REFERENCES

Bülbring E (1937) The standardization of cortical extracts by the use of drakes. J Physiol (London) 89:64–80

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Junkmann K (1955) Über protrahiert wirksame Corticoide. Naunyn-Schmiedeberg's Arch exper Pathol Pharmacol 227:212–213

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Tolksdorf S, Battin ML, Cassidy JW, McLeod RM, Warren FH, Perlman PL (1956) Adrenocortical properties of $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,11,20-trione (Meticorten) and $\Delta^{1,4}$-pregnadiene-11β,17α21-triol-3,20-dione (Meticortelone). Proc Soc Exp Biol Med 92:207–214

N.2.2.1.2 Electrolyte excretion

PURPOSE AND RATIONALE

Mineralocorticosteroids enhance sodium retention and potassium excretion. The sodium excretion in adrenalectomized rats is dose-dependently decreased. This parameter can be used for mineralocorticoid activity of test compounds (Kagawa et al. 1952).

PROCEDURE

Male Sprague-Dawley rats weighing 140–160 g are adrenalectomized. They are maintained on 1% NaCl solution as drinking fluid. On the morning of the fourth postoperative day, food and drinking fluid are withdrawn. On the following day, each rat is given 5 ml water by stomach tube; one hour later 5 ml 0.9% NaCl orally. Test compounds may be injected s.c. in 0.2 ml of vehicle suspension. The rats are lightly anesthetized with ether to induce emptying of the bladder and placed in metabolic cages, 2 rats per cage, 3 cages per dosage group, for 4 h, again anesthetized with ether and removed from the cages. Urine volume is recorded and cages rinsed over the collection cylinders with a distilled water spray. Collections are diluted to 100 ml and appropriate dilutions analyzed for sodium with a flame photometer. Sodium is expressed as percent of excretion of control animals. Desoxy-corticosterone acetate in doses between 1 and 40 µg per rat is used as standard.

EVALUATION

Percent reduction of sodium excretion compared with controls is calculated for each dosage group. Dose-response curves are compared with the dose-response curve of desoxycorticosterone acetate to calculate potency ratios.

MODIFICATIONS OF THE METHOD

Simpson and Tait (1952) measured both urinary sodium and potassium and used the sodium-to-potassium ratio as an index of electrolyte activity of corticoids. Nikisch et al. (1991) infused glucocorticoid-substituted adrenalectomized rats with saline-glucose solution containing aldosterone and measured sodium and potassium concentrations in 1 h fractions of urine. The anti-aldosterone activity was assessed by the ability of test compounds to reverse the aldosterone effect on the urinary Na/K ratio.

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Stafford RO, Barnes LE, Bowman BJ, Meinzinger MM (1955) Glucocorticoid and mineralocorticoid activities of Δ^1-fluorohydrocortisone. Proc Soc Exp Biol Med 89: 371–374

N.2.2 *In vitro* methods

N.2.2.1 Mineralocorticoid receptor binding

PURPOSE AND RATIONALE

Rat kidney receptor preparations and radioactive labeled aldosterone are used to test binding to the mineralocorticoid receptor (Raynaud et al. 1975; Pasqualini and Sumida 1977; Ojasoo and Raynaud 1978).

PROCEDURE

Kidney homogenates of adrenalectomized rats are centrifuged at 0 °C for 10 min at 800 *g* in a buffer solution (10 mM Tris, 0.25 M saccharose, HCl, pH 7.4). Following addition of RU 28 362 at 0.001 mM (to inhibit binding of aldosterone to the glucocorticoid receptor) the supernatant is centrifuged again at 105 000 *g* for 60 min. The supernatant (cytosol) is removed and incubated at 0 °C with ^{3}H-aldosterone (5 nM) and increasing concentrations of test compounds (0–25 000 nM). Non-specific binding is determined in the presence of 1 µM aldosterone.

Free ^{3}H-aldosterone is removed from the incubation medium by the charcoal-dextran technique after 1 h or 24 h of incubation (Raynaud 1978). Following centrifugation, concentration of receptor bound ligand is determined in the supernatant by liquid scintillation counting.

EVALUATION

The following parameters are calculated:

- total binding of ^{3}H-aldosterone
- non-specific binding in the presence of 1 µM aldosterone
- specific binding = total binding – non-specific binding
- % inhibition: 100 – specific binding as percentage of the control value.

Compounds are first tested at a single high concentration (25 000 nM) in triplicate. For those showing more than 50% inhibition a displacement curve is determined using 7–8 different concentrations of compound. Binding potency of compounds is expressed as binding affinity (RBA), relative to the standard compound (aldosterone).

MODIFICATIONS OF THE METHOD

Affinity for the mineralocorticoid receptor was tested in the cytosol of rabbit kidneys (Claire et al. 1993) or rat kidney slices (Funder et al. 1974). It is also useful to use overexpressed human mineralocrticoid receptor.

Monkey kidney COS-1 cells were transfected with plasmids containing the human mineralocorticoid receptor – being cloned by Arriza et al. (1987) – and glucocorticoid receptor for binding studies with various steroids (Rupprecht et al. 1993a,b).

A survey on mineralocorticoid receptor ligands has been given by Sutano and de Kloet (1991).

Specific bioluminescent *in vitro* assays for selecting potential antimineralocorticoids were developed by Jausons-Loffreda et al. (1994).

Wehling (1994) found evidence for a membrane-bound mineralocorticoid receptor in human mononuclear leukocytes.

The human mineralocorticoid receptor was used by Grassy et al. (1997) to study structure-activity relationships of steroids with antimineralocorticoid activity.

Davioud et al. (1996) described synthesis and biological activities of new steroidal diazo ketones as potential photoaffinity labeling reagents for the mineralocorticoid receptor.

Fagart et al. (1997a) recommended [^{3}H-2]-21-diazoprogesterone as a potent photoaffinity labeling reagent for the mineralocorticoid receptor.

Fagart et al. (1997b) proposed a three-dimensional model for antagonism in the human mineralocorticoid receptor.

The biological and clinical relevance of glucocorticoid and mineralocorticoid receptors has been reviewed by Funder (1997).

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Raynaud JP, Ojasoo T, Bouton MM, Philibert D (1979) Receptor binding as a tool in the development of new bioactive steroids. In: Ariëns EJ (ed) Drug Design, Vol VIII, Academic Press, New York, pp 169–214

Rupprecht R, Reul JMHM, van Steensel B, Spengler D, Söder M, Berning B, Holsboer F, Damm K (1993a) Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. Eur J Pharmacol, Mol Pharmacol Sect 247:145–154

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Wehling M (1994) Novel aldosterone receptors: specificity-conferring mechanism at the level of the cell membrane. Steroids 59:160–163

N.2.2.2.2 Transactivation assay for mineralocorticoids

PURPOSE AND RATIONALE

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor acts with hormone responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon (1988). The hormone-dependent transcriptional activation can be determined in tissue culture by transfection of the steroid receptor under investigation and a reporter gene linked to a hormonally responsive promotor into cells. The transactivation assay allows determination of the agonistic and also of the antagonistic activity of a given compound, by either induction or inhibition of reporter gene activity (Fuhrmann et al. 1992).

PROCEDURE

Vector construct

The expression plasmid pRShMR containing the full-length coding sequence of the mineralocorticoid receptor expressed from the long terminal repeat of the Rous sarcoma virus is prepared according to Arriza et al. (1987). The pMMTV-CAT plasmid containing the mouse mammary tumor virus promotor linked to a chloramphenicol acetyltransferase gene is prepared according to Cato et al. (1986).

Cell culture and transfections

CV-1 cells and COS-1 cells for transient infections are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 4 mmol/L L-glutamine, penicillin and streptomycin. Stable and transient transfections are performed using Lipofectin reagent (Gibco BRL) according to a procedure recommended by Felgner and Holm (1989). Stable transfections are carried out according to Fuhrmann et al. (1992). For transient transfection, 1×10^6 COS-1 or CV-1 cells, respectively, are plated onto 100-mm dishes one day prior to transfection. Cells are typically about 80% confluent after 24 h. Before transfection,

cells are washed twice with 1 ml Opti-MEM (Gibco BRL) per dish. For each dish, 5 µg pRShMR (hMR expression plasmid) and 5 µg pMMTV-CAT are diluted with 1 ml Opti-MEM; in addition, 50 µg Lipofectin Reagent is diluted with 1 ml Opti-MEM. Next, DNA and Lipofectin Reagent dilutions are combined in a polystyrene snap-cup tube to obtain 2 ml of transfection solution per dish, gently mixed, incubated at room temperature for 5 min, and added to the washed cells. After 5 h the transfection solution is replaced by 6 ml DMEM supplemented with 10% fetal calf serum.

To study the effect of the test hormones, transiently transfected cells are trypsinized, pooled and replated onto 60-mm dishes at a density of 4.5×10^5 per dish 24 h after transfection. Stably transfected cells are seeded onto 6-well dishes (1×10^5 cells per dish). Cells are cultured in medium supplemented with 3% charcoal-stripped fetal calf serum and the appropriate hormones for 48 h. As negative control for the reporter gene induction, cells are cultured with 1% ethanol. Transactivation assays with transiently or stably transfected cells are carried out at least three times.

CAT assay

Transiently transfected cells and stably transfected cells are disrupted by freezing (ethanol/dry ice bath) and thawing (37 °C water bath) three times. Protein concentrations of the cell extract are determined according to Bradford (1976). The CAT assay is performed according to Gorman et al. (1982).

EVALUATION

CAT activity is calculated as percent conversion from chloramphenicol to acetylated chloramphenicol. Concentration-response curves for CAT induction are established to demonstrate the potency of the test hormone. Aldosterone (10^{-10} to 10^{-6} mol/l) serves as standard.

For antimineralocorticoid activity, CAT activity in the presence of 10^{-8} mol/l aldosterone is set as 100% and relative CAT activity is calculated as percentage of this value. Concentration-response curves for CAT inhibition are established with increasing concentrations of the antihormone.

CRITICAL ASSESSMENT OF THE METHOD

See N.2.1.1.2.

MODIFICATIONS OF THE METHOD

White et al. (1994) described a simple and sensitive high-throughput assay for steroid agonists and antagonists. A DNA cassette, containing a synthetic steroid-inducible promotor controlling the expression of a bacterial chloramphenicol acetyltransferase gene (GRE5-CAT), was inserted into an Epstein-Barr virus episomal vector, which replicates autonomously in primate and human cells. This promotor/reporter system was used to generate two stably transfected human cell lines. In the cervical carcinoma cell line HeLa, which expresses high level of glucocorticoid receptor, the GRE5 promotor is inducible over 100-fold by the synthetic glucocorticoid dexamethasone. In the breast carcinoma cell line T47D, which expresses progesterone and androgen receptors, the GRE5 promotor is inducible over 100-fold by either progesterone or dihydrotestosterone. These cell lines can be used to screen large numbers of natural and synthetic steroid agonists and antagonists in microtiter wells directly using a colorimetric chloramphenicol acetyltransferase assay.

Rupprecht et al. (1993a,b) examined the functional agonistic and antagonistic activity of several steroids by co-transfecting human mineralocorticoid or human glucocorticoid receptor expression vectors, together with a mouse mammary tumor virus-luciferase (MTV-LUC) reporter gene into the human neuroblastoma cell line SK-N-MC. Transfections were performed using an electroporation system (Biotechnologies and Experimental Research, San Diego, CA).

Lombès et al. (1994) used a cotransfection assay in CV-1 cells to study the discrimination of aldosterone from natural and synthetic glucocorticoids by the human mineralocorticoid receptor. Cells were transfected by the calcium phosphate method with pRShMR, a plasmid that contains the entire coding sequence of the human mineralocorticoid receptor; pFC31Luc, which contains the mouse mammary tumor virus (MMTV) promotor driving the luciferase gene; pCH110 encoding the β-galactosidase as an internal transfection control; and pSP72 as plasmid carrier.

Lim-Tio et al. (1997) studied the determinants of specificity of transactivation by the mineralocorticoid or glucocorticoid receptor in three cell lines: CV-1 cells; a porcine renal epithelial cell line, LLC-PK1; a pig kidney cell strain, and RN33B; a neuronal medullary raphe cell line. The reporter gene used was MMTV-LUC (the long terminal repeat of the mouse mammary tumor virus promotor linked to the luciferase reporter gene). RSV-CAT (Rous sarcoma virus promotor-chloramphenicol acetyl transferase gene) was used as internal control for transfection efficacy.

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N.2.2.3 Antimineralocorticoid activity

N.2.2.3.1 Electrolyte excretion

PURPOSE AND RATIONALE

The method is based on mineralocorticoid activity of desoxycorticosterone acetate reversed by simultaneous administration of antimineralocorticoids.

PROCEDURE

Male Sprague-Dawley rats weighing 140–160 g are adrenalectomized and maintained on 1% NaCl solution as drinking fluid. On the morning of the fourth postoperative day, food and drinking fluid are withdrawn. On the following day, each rat is given 5 ml water by stomach tube; one hour later it is given 5 ml 0.9% NaCl orally and injected with 40 µg desoxycorticosterone acetate. At a separate site, the antagonistic test compound is injected s.c. in 0.2 ml of vehicle suspension. Spironolacton 50 µg and 500 µg is injected as the standard to separate reference groups. The rats are placed in metabolic cages and lightly anesthetized with ether to induce emptying of the bladder, 2 rats per cage, 3 cages per dosage group, for 4 h, and again anesthetized with ether before being removed from the cages. Urine volume is recorded and cages rinsed over the collection cylinders with a distilled water spray. Collections are diluted to 100 ml and appropriate dilutions analyzed for sodium and potassium with a flame photometer.

EVALUATION

The amount of excreted water, sodium and potassium per 100 g rat is calculated. The product of water volume and sodium excretion is divided by the potassium excretion. This quotient is compared with the values of untreated adrenalectomized rats and animals treated with desoxycorticosterone acetate only.

MODIFICATIONS OF THE METHOD

Antimineralocorticoid activity of spironolactone and its analogues has been tested in adrenalectomized golden hamsters treated simultaneously with daily injections desoxycorticosterone acetate over a period of 3 weeks (Vogel 1965). Mean survival of adrenalectomized animals of 4.7 days was prolonged to 13.5 days by daily subcutaneous injections of 2 mg desoxycorticosterone acetate. Additional injection of 0.5 or 1.0 mg spironolactone reduced the survival time.

Losert et al. (1985) tested the ability of several steroids with progestogenic potency to inhibit the renal actions of aldosterone in adrenalectomized, glucocorticoid-treated rats. The rats were continuously infused with an isotonic solution of low sodium content (0.05% NaCl + 5.2% glucose, 3 ml/rat/h) supplemented with d-aldosterone (1 µg/kg/h) resulting in a long-lasting reduction in renal sodium excretion, increase in renal potassium excretion and hence decrease in the urinary Na/K ratio. The test drugs were administered either subcutaneously or orally 1 h before start of infusion. The antimineralocorticoid activity was judged by the increase in the aldosterone-lowered Na/K ration in urine collected at hourly intervals up to 21 h.

De Gasparo et al. (1987) evaluated epoxy-spironolactone derivatives for their antimineralocorticoid activity (Kagawa test) and their antiandrogenic and progestogenic side effects *in vitro* and *in vivo*.

Gómez-Sánchez et al. (1990) reported the effect of intra-cerebroventricular infusion of mineralocorticoid antagonists on the hypertension in rats produced by chronic subcutaneous administration of aldosterone.

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N.3 Ovarian hormones

N.3.0.1 Castration of female rats

PROCEDURE

Ovariectomy is performed in immature female rats weighing less than 60 g. The animal is slightly anesthetized with ether. A single transverse incision is made in the skin of the back. This incision can be shifted readily from one side to the other so as to lie over each ovary in turn. A small puncture is then made over the site of the ovary, which can be seen through the abdominal wall, embedded in a pad of fat. The top of a pair of fine forceps is introduced and the fat around the ovary is grasped, care being taken not to rupture the capsule around the ovary. The tip of the uterine horn is then crushed with a pair of artery forceps and the ovary, together with the fallopian tube, is removed with a single cut by a pair of fine scissors. Usually, no bleeding is observed. In older rats, the tip of the uterine horn may be ligated and the ovary removed distally from the ligature. The ovary of the other side is removed in the same way. The skin wound is closed by one or two clips. The animal recovers immediately. With some skill, the operation can be performed very rapidly.

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N.3.1 Estrogens

N.3.1.1 *In vitro* methods

N.3.1.1.1 Estrogen receptor binding

PURPOSE AND RATIONALE

This assay is used to estimate estrogenic activity and potency of test compounds. Estradiol-17β is used as the reference compound. Estrogen receptors are prepared from mouse uteri or from human endometrium. Measurements of association rates and dissociation rates at different temperatures allow evaluation of relative binding affinities (Bouton and Raynaud 1977, 1978). Binding to the cytosolic and the nuclear fraction is measured.

PROCEDURE

Cytosol preparation. Uteri from 18-day old female Swiss mice are removed and homogenized at 0 °C in 1 : 50 (w/v) of 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose buffer in a conical homogenizer. Human endometrium from menopausal women is frozen within 2 h of hysterectomy and stored in liquid nitrogen until use. The frozen endometrium is pulverized and homogenized in 1 : 5 (w/v) Tris-sucrose buffer. Homogenates are centrifuged for 1 h at 105 000 *g*.

Dextran-coated charcoal (DCC) adsorption technique. Binding is measured as follows. A 100 µl aliquot of incubated cytosol is stirred for 10 min at 0 °C in a micro-titer plate with 100 µl of Dextran-coated charcoal (DCC) suspension (0.625% Dextran 80 000, 1.25% charcoal Norit A) and then centrifuged for 10 min at 800 *g*. The concentration of bound steroid is determined by measuring the radioactivity in a 100 µl aliquot of supernatant.

Determination of specific binding in mouse uterus cytosol as a function of steroid concentration, incubation time and temperature. Triplicate aliquots of 125 µl of cytosol are incubated with 5 or 25 nM labelled steroid (estardiol-17ß) either for 2 or 24 h at 0 °C or for 2 or 5 h at 25 °C in the absence (total binding) or presence (non-specific binding) of a 100-fold excess of radio-inert steroid. Bound steroid is measured by DCC adsorption.

Measurement of association rate at 0 °C. 1 or 5 nM concentration of labelled steroid is added to cytosol maintained at 0 °C. Every 5 min for 1 h after addition of the labelled steroid, a 100 µl aliquot is transferred into a 5 000 nM radio-inert steroid solution in a microtiter plate to stop the reaction. Bound radioactivity is determined by DCC adsorption.

Measurement of dissociation rate at 25 °C. Dissociation rate is measured by the isotopic dilution technique. Radio-inert steroid (2 500 nM) is added to crude cytosol previously incubated with 5 nm labelled steroid for 15 h at 0 °C. After different times of incubation at 25 °C, 100 µl samples are treated with DCC at 0 °C in order to determine bound radioactivity. Specific binding is evaluated by subtracting non-specific binding from total binding.

Nuclear uptake. Homogenate samples (0.5 ml; 2 uteri/0.5 ml) are incubated with 2.5, 5 or 25 nM labelled steroid for 5 or 60 min at 25 °C, then cooled on ice and centrifuged at 800 *g* for 10 min at 0 °C. The pellet (crude nuclei) is washed 3 times with 1 ml Tris-sucrose buffer, dissolved in 0.5 ml Soluene (Packard) and counted.

Nuclear extract preparation. After incubation with 25 nM labelled steroid for 1 h at 25 °C, the crude nuclei are resuspended in 0.5 ml Tris-sucrose buffer, to which 50 µl of 4 M KCl are added. The suspension is stirred (Vortex), left for 30 min at 0 °C and then centrifuged for 30 min at 105 000 *g*. The radioactivity in a 100 µl supernatant sample (nuclear extract) is counted.

EVALUATION

For calculation of **relative binding affinity**, the percentage of radioligand bound in the presence of competitor compared to that bound in its absence is plotted against the concentration of unlabeled competing steroid. A standard curve for the competition of the unlabeled radioligand is constructed with the use of 9–10 concentrations; 5 or 6 concentrations of the competitor are tested. These are chosen to provide a linear portion of the semilog plot which crosses the point of 50% competition. From this plot, the molar concentration of unlabeled radioligand or steroid competitor that reduces radioligand binding by 50% is determined.

The relative affinity of a test compound is established as the ratio of unlabeled radioligand concentration to competitor concentration, at 50% competition. This ratio is multiplied by 100.

Association rate (k_{+1}) is calculated by the slope of the line

$$k_{+1}\, t = (2.3 / E_0 - R_0) \log (E\, R_0 / R\, E_0)$$

where E_0 and E represent free steroid and R_0 and R free receptor at time $t = 0$ and time t, respectively.

Dissociation rate (k_{-1}) is calculated from the slope of the line

$$k_{-1} = -2.3 \log B / B_0$$

where B_0 and B represent bound steroid at time $t = 0$ and time t, respectively.

MODIFICATIONS OF THE METHOD

Sheep uteri were used for the preparation of cytosol by Shutt and Cox (1972).

Pons et al. (1990) described receptor binding of estrogens and anti-estrogens in the estrogen receptor-positive breast cancer cell line MCF-7, using the firefly luciferase assay (Brasier et al. 1989) as endpoint.

Ludwig et al. (1990) described a microliter well assay for quantitative measurement of estrogen receptor binding to estrogen-responsive elements.

Hwang et al. (1992) studied the use of tetrahydrochrysenes, inherently fluorescent, which are high-affinity ligands for the estrogen receptor.

Estrogen receptor binding has also been used to study the mechanism of action of non-steroidal anti-estrogens (Jordan et al. 1977; Wakeling and Slater 1980; Astroff and Safe 1988).

Sequence and expression of human estrogen receptor complementary DNA has been reported by Greene et al. (1986).

Solution structure of the DNA-binding domain of the estrogen receptor has been described by Schwabe et al. (1990).

Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β has been reported by Tremblay et al. (1997).

Nichols et al. (1998) showed that agonists and antagonists differently position the C-terminus of the ligand-binding domain (helix 12) and the F domain of the estrogen receptor.

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N.3.1.1.2 Transactivation assay for estrogens

PURPOSE AND RATIONALE

Transient transfection of a suitable cell culture with an estrogen-responsive-element (ERE)-controlled reporter gene has become a convenient assay for estrogens.

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor interacts with hormone responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon 1988).

The assay in the estrogen-receptor-positive human breast cancer cell line MCF-7, described by Meyer et al. (1994) as a rapid luciferase transfection assay

for transcription activation of estrogenic drugs, has been used for evaluation of synthetic estrogen antagonists by von Angerer et al. (1994, 1995), Biberger and von Angerer (1996).

PROCEDURE

MCF-7 cells used for transfection are grown in Dulbecco's Modified Essential Medium supplemented with 10% fetal calf serum, 100 U penicillin, 100 µg streptomycin and 150 mg L-glutamine in 500 ml of medium without phenol red. Shortly before confluence, the cells are washed with 10 ml of PBS. Cells are gently shaken for a few seconds with trypsin-EDTA solution (4 ml) and after removal of the solution incubated for 2 min at 37 °C. After addition of 10 ml of medium, the cell suspension (0.5 ml/well) is transferred to 6-well plates containing 2 ml of medium. Cells are grown until the density of the monolayer is about 50% (1–2 days) before 2 µg of the luciferase reporter plasmid EREwtc luc, harboring the luciferase gene from Photinus pyralis, are added. For a successful transfection, it is necessary to generate a very fine precipitate of the DNA by subsequent dilution with 45% water, 5% M $CaCl_2$, and 50% HBS buffer and continuous shaking. After 20 min at room temperature, an opalescent solution should be obtained.

After addition of the DNA solution, the medium is removed and the cells are washed with 2 ml of PBS, followed by treatment with glycerol (15% in PBS) for 2 min. After washing with PBS, fresh medium containing the test substances is added. The maximum of luciferase expression is reached 18 h after addition of the transfection solution. At this time, the medium is removed and cells are washed with PBS. Cell lysis and quantification of luminescence is performed according to the procedure described in the luciferase assay system E1 500 of PROMEGA (Serva, Heidelberg, Germany). Luminescence is measured in a luminometer Lumat LB (Berthold, Wildbad, Germany) as relative light units, which are converted into fg luciferase by a calibration curve.

EVALUATION

Dose response curves of luciferase activity after induction by the test substances and estradiol as control (10^{-14} to 10^{-9} M) are established.

CRITICAL ASSESSMENT OF THE METHOD

See N.2.1.1.2.

MODIFICATIONS OF THE METHOD

With the same method as for MCF-7 cells, von Angerer et al. (1994), Biberger and von Angerer (1996) transfected HeLa-cells using the estrogen expression receptors HE0, HEG0, HE15 and HEG19, together with the receptor plasmid EREwtc luc.

Bergmann et al. (1994) determined estrogenic activity by a transient transfection assay in ER-deficient CHO cells transfected with an expression vector encoding the estrogen receptor using an estrogen-responsive reporter gene construct, $(ERE)_2$-TATA-CAT, containing two estrogen response elements linked to a TATA promotor and the chloramphenicol transferase reporter gene.

For assessing environmental chemicals for estrogenicity, Shelby et al. (1996) recommended a transcriptional activation assay in ER-transfected HeLa cells using the estrogen responsive reporter, ERE81CAT, and the pRSV vector containing the mouse ER cDNA without the neomycin resistance cassette. Triplicate samples for each hormone concentration were harvested at 28 h post-transfection and assayed for CAT protein using the CAT-ELISA kit (Boehringer Mannheim).

Several authors used yeast for assays of estrogenicity (McDonnell et al. 1991; Pierrat et al. 1992; Kohno et al. 1994; Bush et al. 1996; Tran et al. 1996; Odum et al. 1997)

Pierrat et al. (1992) constructed yeast strains in which the *Saccharomyces cerevisiae* URA3 gene is induced by the human estrogen receptor. Promotor sequences required for both basal and activated transcription of URA3 were replaced with estrogen-response elements positioned upstream of the native TATA box. These constructs were integrated at the TRP1 locus of a yeast strain in which the natural URA3 gene has been deleted and the integrants were transformed with shuttle plasmids expressing wild-type or truncated derivatives of human estrogen receptor. Transformants were assayed for growth on uracil-deficient medium plus or minus estradiol, for resistance to 5-fluoroorotic acid, and for activity of orotidine-5'-monophosphate decarboxylase, the product of the URA3 gene.

Tran et al. (1996) used the yeast strain ER(wt) expressing human estrogen receptor and an estrogen-sensitive reporter to characterize the estrogenic or antiestrogenic activities of polynuclear aromatic hydrocarbons.

Gaido et al. (1997) described a yeast-base steroid hormone receptor gene transcription assay to evaluate estrogenic and androgenic activity. The yeasts contain two separate plasmids: (1) an expression plasmid which contains the CUP1 metallothionein promotor fused to the human estrogen receptor cDND, and (2) a receptor plasmid carrying two estrogen response elements or a reporter plasmid carrying two copies of a progesterone/androgen responsive element upstream of the structural gene for β-galactosidase.

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N.3.1.1.3
Estrogen dependent cell proliferation

PURPOSE AND RATIONALE

Specific cell proliferation induced by estrogens is the principle of these assays. Some human breast cancer cell lines, such as MCF-7 and T47-D cells, respond to estrogens with proliferation. This effect has been used for assays of estrogenic and antiestrogenic activity (Scholl et al. 1983; Miller and Katzenellenbogen 1983, Thompson et al. 1984; Palkowith et al. 1997; Zacharewski 1997).

PROCEDURE

MCF-7 breast adenocarcinoma cells are maintained in MEM (Minimal Essential Medium), minus phenol red (which is estrogenic at high concentrations) supplemented with 10% fetal bovine serum (FBS), 2 µM L-glutamine, 1 µM sodium pyruvate, 10 µM HEPES, nonessential amino acids and 1 mg/ml bovine insulin. Ten days prior to assay, MCF-7 cells are switched to maintenance medium supplemented with 10% dextran-coated FBS in place of 10%FBS to deplete internal stores of steroids. MCF-7 cells are removed from maintenance flasks using cell dissociation medium (Ca^{2+}/Mg^{2+} free HBSS, (phenol red-free) supplemented wit 10 µM HEPES and 2 nM EDTA). Cells are washed twice with assay medium and adjusted to 80 000 cells/ml. Approximately 100 µl (8 000 cells) is added to flat-bottomed microculture wells and incubated at 37 °C in a 5% CO_2 humidified incubator for 48 h to allow for cell adherence and equilibration transfer. Serial dilutions of test compounds, or DMSO as a diluent control, are prepared in assay medium and 50 µl transferred to triplicate microcultures, followed by 50 µl assay medium for a final volume of 200 µl. After an additional 48 h incubation, microcultures are pulsed with 1 µCi [^{3}H]thymidine (specific activity 6.7 Ci/mmol) for the last 5 4–6 h of culture and the assay terminated by freezing at –70 °C. Microcultures are then thawed and harvested using a Skatron semi-automatic cell harvester. Samples are counted by liquid scintillation using a Wallace BetaPlate β-counter.

EVALUATION

Plotting of dpm versus compound concentration is used to determine the half maximal effective concentrations EC_{50} or inhibitory concentration IC_{50}.

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N.3.1.2 *In vivo* methods

N.3.1.2.1 Vaginal cornification

PURPOSE AND RATIONALE

This is an early bioassay for estrogenic activity based on epithelial proliferation. The Allen-Doisy test for vaginal cornification in rodents (Allen and Doisy 1923) is based on the observations of Stockard and Papanicolaou (1917), who first reported the cyclic vaginal cornification in guinea pigs.

PROCEDURE

Immature female Sprague-Dawley rats weighing about 55 g are ovariectomized. They are kept for about one week on standard laboratory diet and water ad libitum. The test compounds are administered orally or subcutaneously in 0.5% solution of carboxymethyl-cellulose or in cotton seed oil injected at several doses to groups of 10–20 rats. Doses of 0.02, 0.1, and 0.5 µg estradiol per animal are used as standard. The compounds are dosed e.g. twice daily on two following days at 10:00 A.M. and 5:00 P.M. At 5:00 P.M. of the third day and at 10:00 A.M. of the fourth day vaginal smears are taken using cotton swabs moistened with saline. The smears are transferred to a glass slide and stained for 10 min with 5% aqueous methylene blue solution. They are evaluated microscopically according to the following scores:

0 diestrus smear, mainly leukocytes, few epithelia cells
1 mixture of leukocytes and epithelial cells
2 proestrus smear, nucleated or nucleated plus cornified cells
3 estrus smear, cornified cells only.

Only animals showing score 2 or 3 are considered to be positive.

EVALUATION

The number of positive animals in each dosage group is recorded. ED_{50} values can be calculated and compared with the standard estradiol-17β.

MODIFICATIONS OF THE METHOD

The sensitivity of the assay is increased by local applications of estrogens into the vagina of castrated rats or mice (Emmens 1969).

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N.3.1.2.2 Uterus weight

PURPOSE AND RATIONALE

This is an early bioassay for estrogenic activity based on myometrial/endometrial proliferation. Repeated administration of estrogens induces a dose-dependent increase of uterine weight in castrated female rats.

PROCEDURE

Immature female Sprague-Dawley rats weighing about 55 g are ovariectomized. Groups of 5–10 animals are injected daily with several doses of the test compound or the standard (estradiol 0.03 to 0.06 µg per animal s.c.) for seven days. The test compound is administered orally or subcutaneously in 0.5% solution of carboxymethylcellulose or in cotton seed oil. Controls receive the vehicle only. On the 8th day, the animals are sacrificed and uterine weights determined.

EVALUATION

Using at least two doses of test compound and standard each, dose-response curves are established and potency ratios calculated.

MODIFICATIONS OF THE METHOD

Rubin et al. (1951) used albino mice 23–25 days of age weighing approximately 8 g. The mice are given subcutaneous injections once daily for 3 days of an oil solution of the hormone. Twenty-four hours after the last injection, the animals are sacrificed and uterine and body weights recorded. The uterine ratio is calculated by dividing uterine weight in milligrams by body weight in grams, multiplied by 100 (relative uterine weight). Two groups receive a standard preparation and two groups the unknown.

Bhakoo and Katzenellenbogen (1977) showed that one of the earliest biosynthetic tissue responses after estrogen binding in the rat uterus, the synthesis of a specific uterine protein, called 'induced protein', is antagonized by progesterone.

In addition to uterus weight, Branham et al. (1993) studied luminal and glandular epithelium height in cross-sections of the uterine horns of rats by histological means.

Uterine peroxidase activity was proposed as a marker for estrogen action by Lyttle and DeSombre (1977), Astroff and Safe (1991).

Odum et al. (1997) compared the rodent uterotrophic assay with a yeast estrogenicity assay.

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N.3.1.2.3 Chick oviduct method

PURPOSE AND RATIONALE

This is an early bioassay for estrogenic activity based on proliferation of the oviduct. The weight of the oviduct of young chicken is increased dose-dependently by natural and synthetic estrogens (Tullner and Hertz 1956).

PROCEDURE

Seven-day old pullet chicks are injected subcutaneously twice daily with solutions of the test compound in various doses for 6 days. Doses between 0.02 and 0.5 μg estradiol-17β per animal serve as standard. Six to 10 chicks are used for each dosage group. On the day after the last injection, the animals are sacrificed and weight of the body and oviduct determined.

EVALUATION

The ratio oviduct weight/body weight is calculated for each animal (relative weight). Mean values are plotted as dose-response curves in order to calculate potency ratios.

MODIFICATION OF THE METHOD

The assay can be used for evaluation of anti-estrogenic activity using simultaneous injection of 0.6 μg estradiol-17β or stilbestrol and the inhibitor, e.g., progestagens, and calculation of the percentage of oviduct weight in estrogen/antiestrogen treated animals versus the values of estrogen treatment only.

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N.3.1.3 Anti-estrogenic activity

N.3.1.3.1 Antagonism of uterus weight increase due to estrogen treatment

PURPOSE AND RATIONALE

These assays are modifications of estrogen bioassays. The increase of uterine weight in castrated female rats induced by estradiol can be antagonized by anti-estrogenic compounds.

PROCEDURE

Immature female Sprague-Dawley rats weighing about 55 g are ovariectomized. Groups of 5–10 animals are injected daily for seven days with estradiol 0.03 to 0.06 µg per animal s.c. and various doses of the test compound or estradiol alone. The test compound is administered in 0.5% solution of carboxymethylcellulose or in cotton seed oil either orally or injected subcutaneously. On the 8th day, the animals are sacrificed and the uterine weights determined.

EVALUATION

Mean values of each group are calculated. The anti-estrogenic effect is expressed as percent reduction of estrogen-stimulated uterine weight by test compounds compared to rats treated with estradiol alone.

MODIFICATIONS OF THE METHOD

Three-week old female NMRI mice can be used for determination of uterus weight (Lerner et al. 1958).

The inhibition by anti-estrogens of vaginal cornification induced by estradiol can be used as parameter.

The chick oviduct weight can be adapted for the assay of anti-estrogenic activity (Tullner and Hertz 1956).

Castrated immature male rats show an increase in weight of the seminal vesicles when treated with 20 µg estradiol for 5 days, the effect is counteracted by anti-estrogenic steroids (Byrnes et al. 1953).

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N.3.1.3.2 Aromatase inhibition

PURPOSE AND RATIONALE

Estrogen synthesis by aromatase occurs in various tissues including estrogen dependent tumors. Specific aromatase (estrogen synthetase) inhibitors are potentially useful therapeutic agent for estrogen dependent tumors, such as some breast cancers. 4-Hydroxyandrostandione (4OHA) inhibits aromatase completely but also causes destruction of the enzyme. Test compounds can be evaluated both *in vitr*o (Häusler et al. 1989) and *in vivo* (Geelen et al. 1991). Tests are based on stimulation of estrogen biosynthesis by LH and inhibition of this effect by aromatase inhibitors.

PROCEDURE

For *in vitro* experiments, ovarian tissue from adult golden hamsters (*Mesocricetus auratus*) is used. Estrus cycle is monitored for at least 3 consecutive 4-day estrus cycles prior to the experiment. The experiments for evaluating inhibitor effects are performed with ovaries obtained from animals sacrificed on day 4 (proestrus), at the time of preferred estrogen synthesis. The ovaries are excised, freed from adhering fat tissue and quartered. The quarters are transferred into plastic incubation flasks with 2 ml of Krebs-Ringer-bicarbonate (KBR) salt solution, pH 7.6, containing 8.4 mM glucose. The flasks are gassed with O_2/CO_2 (95%/5%), tightly closed and placed in a shaker/water bath (37 °C) for incubation of the fragments. The in-

cubation media are replaced with fresh KBR after preincubation for 1 h.

The ovaries are further incubated for 4 h in the presence or absence of ovine LH (100 ng/ml) and inhibitors to be tested. 4-OH-Androstendione is used as standard in concentrations between 0.33 and 330 µM/L. At the end of the experiment, the incubation media are removed and centrifuged. In the supernatant, estrogen, progesterone and testosterone are determined by radioimmunoassays.

EVALUATION

The results are expressed as percentage of inhibition relative to control incubations containing 100 ng/ml LH for estrogen stimulation in the absence of inhibitor. Statistical analyses are performed using Dunnetts's *t*-test.

MODIFICATIONS OF THE METHOD

Geelen et al. (1991) determined the *in vivo* aromatase activity of test compounds in hypophysectomized rats treated with the estrogen precursor dihydroepiandrosterone sulfate, using the inhibition of cornification of vaginal epithelium and estradiol levels in plasma as parameters. Furthermore, aromatase activity was determined in homogenized ovaries. To the supernatant, [1β-^{3}H]androst-4-ene-3,17-dione was added. Incubation was performed with a NADPH generating system for 1 h at 37 °C. Incubations were terminated by placing the tubes on ice followed by an extraction with chloroform. After phase separation by centrifugation, the aqueous phase was diluted with an equal volume of dextran coated charcoal suspension. Following centrifugation, the ^3H_2O content was determined by liquid scintillation counting. The amount of ^3H_2O is a measure of the amount of estrogen produced.

Zaccheo et al. (1989) studied the antitumor activity of aromatase inhibitors in rats with 7,12-dimethylbenzanthracene (DMBA)-induced tumors.

Wouters et al. (1993) studied the inhibition of aromatase activity in FSH-stimulated rat granulosa cells by vorozole, a selective, non-steroidal aromatase inhibitor.

Suzuki et al. (1996) described changes in prostate volume and histopathological findings in androstenedione-treated castrated beagle dogs as a bioassay for an aromatase inhibitor.

Takahashi et al. (1997) tested the inhibition of aromatase activity in the microsomes from fresh human placentae by the amount of tritiated water released from [1β-^{3}H]androstenedione. For biological proof, the dose-dependent suppression of androstenedione-induced uterine hypertrophy in immature rats was measured.

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N.3.1.3.3 Anti-estrogenic effect on MCF-7 breast cancer cells

PURPOSE AND RATIONALE

The MCF-7 cell line, derived from a pleural effusion of a malignant breast cancer, is a widely studied model for hormone-dependent human breast cancer. These cells contain functional estrogen receptors and show a pleiotropic response to estrogen which can be used to evaluate antiestrogenic effects (Miller and Katzenellenbogen 1983; Scholl et al. 1983; Thompson et al. 1988). These cells proliferate and invade through an artificial basement membrane. Antiestrogens reduce both the proliferation and invasiveness of these cells.

PROCEDURE

MCF-7 cells are maintained in T75 flasks in IMEM (Biofluids, Rockville, MD) supplemented with 2 mM glutamine and 10% fetal bovine serum. To deplete estrogen, the cells are passaged for at least 2 weeks in IMEM supplemented with 5% calf serum which has been treated sequentially with sulfatase and dextran coated charcoal (DCC) to remove endogenous estrogen.

Estrogen and antiestrogen treatment

Cells are trypsinized, reseeded in tissue culture dishes (1×10^6 cells/10-cm-diameter Falcon dish), and allowed to adhere overnight in a humidified incubator (37 °C, 5% CO_2, 95% air). The cells are treated the next day with either 17β-estradiol (10^{-9} M) or the antiestrogen or 0.1% ethanol alone. Four days later,

the cells are harvested with trypsin, washed twice in IMEM containing 0.1% BSA, counted with a Coulter cell counter and tested for chemotaxis and chemo-invasion activities.

Chemoinvasion assay

Boyden chambers are used (Albini et al. 1987). Polycarbonate filters (12-µm pore, polyvinylpyrrolidone-free, Nucleopore) are coated with matrigel (25 µg/filter), a mixture of basement membrane components (Kleinman et al. 1986), which is dried and then reconstituted at 37 °C into a solid, even layer over the surface of the filter. Fibroblast-conditioned medium, obtained by incubating confluent NIH-3T3 cells for 24 h with IMEM, is used as the chemoattractant. Cells are harvested with trypsin, washed twice with BSA/IMEM, and added to the top chamber (300 000 cells/chamber). Chambers are incubated in a humidified incubator at 37 °C in 5% CO_2 in air for 6, 9, or 12 h. The cells which have traversed the matrigel and attached to the lower surface of the filter are stained with Diff-Quick (American Scientific Products) and quantitated electronically with the Optimax V image analyzer.

Chemotaxis assay

Chemotaxis assays are performed as described for the chemoinvasion studies with the single exception that the filter surfaces are coated with 5 µg collagen IV instead of the layer of matrigel. This coats the interstices of the filters but does not form a barrier over the surface. Chemotaxis assays are performed in parallel to the chemoinvasion assays using the same cells and conditioned medium.

EVALUATION

One-way analysis of variance is performed on the data from each experiment.

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N.3.2
Progestional activity

N.3.2.1
In vitro methods

N.3.2.1.1
Gestagen receptor binding

PURPOSE AND RATIONALE

Progesterone receptor may be obtained from uterine tissue or cultured cells. Uteri from estrogen primed rabbits (Ojasoo and Raynaud 1978; Boonkasemsanti et al. 1989; Phillips et al. 1990; Cook et al. 1992), castrated and estrogen treated mice or rats (Philibert and Raynaud 1977; Li et al. 1997), MCF-7 cells derived from human breast tumor (Bergink et al. 1983; Kloosterboer et al. 1988a,b; Kloosterboer et al. 1994), breast cancer T47D cells (Meyer et al. 1990), the quail fibroblast cell line QT6 (Schowalter et al. 1991) or human uteri obtained after hysterectomy (Jänne et al. 1976; Pollow et al. 1989a,b, 1992). Tritium labeled progesterone or R 5020 ([6,7-^{3}H]17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione), are used as ligands (Moguilewsky and Raynaud 1979).

PROCEDURE

Relative binding affinities. Human uteri obtained after hysterectomy are snap frozen in liquid nitrogen and stored at –80 °C until use. For cytosol preparations, uterine tissues are minced and homogenized with an Ultra-Turrax at 0–4 °C in ice-cold buffer composed of 10 mM KH_2PO_4, 10 mM K_2HPO_4, 1.5 mM EDTA, 3 mM NaN_3, 10% glycerol, pH 7.5 (PENG buffer). The homogenates are then centrifuged at 105 000 *g* at 4 °C for 30 min. The supernatant is taken as cytosol. The cytosol preparations are incubated with ^{3}H-R 5020 as radioligand at a concentration of 8 nmol/l and increasing concentrations (1×10^{-10} to 1×10^{-5} mol/l) of the competitor steroids overnight at 4 °C. Then, unbound steroids are adsorbed by incubating with 0.5 ml of DCC (0.5% Norit A, 0.05% dextran T400 in PENG buffer) for 10 min at 4 °C. After centrifugation (10 min at 1 500 *g* at 4 °C) 0.5 ml of the supernatant is withdrawn and counted for radioactivity.

Association rate and dissociation rate are determined as described for the estrogen receptor.

EVALUATION

For calculation of **relative binding affinity**, the percentage of radioligand bound in the presence of competitor compared to that bound in its absence is plotted

against the concentration of unlabeled steroid. A standard curve for the unlabeled radioligand (progesterone) is constructed with of 9–10 concentrations; 5 or 6 concentrations of the competitor are tested. The molar concentrations of unlabeled radioligand and steroid competitors that reduce radioligand binding by 50% are determined. The ratio of unlabeled radioligand and competitor for 50% competition multiplied by 100 is calculated for relative binding affinity.

Association rate (k_{+1}) is calculated by the slope of the line

$$k_{+1}\, t = (2.3 / E_0 - R_0) \log (E\, R_0 / R\, E_0)$$

where E_0 and E represent free steroid and R_0 and R free receptor at time $t = 0$ and time t, respectively.

Dissociation rate (k_{-1}) is calculated from the slope of the line

$$k_{-1} = -2.3 \log B / B_0$$

where B_0 and B represent bound steroid at time $t = 0$ and time t, respectively.

MODIFICATIONS OF THE METHOD

For screening procedures homogenates of rabbit uteri may be used (Philibert et al. 1977).

The binding of the progesterone agonist R 5 020 and of the progesterone antagonist RU486 to the progesterone receptor from calf uterus was characterized by Hurd and Moudgil (1988).

A high affinity ligand and novel photoaffinity labeling reagent for the progesterone receptor ([^{3}H]DU41 165) was described by Pinney et al. (1990).

Different DNA-binding properties of the calf uterine estrogen and progesterone receptors were explained by different dimerization constants (Skafar 1991).

Mutations of the progesterone receptor were found to be responsible for species specificity and have been used for evaluation of agonistic and antagonistic activity (Benhamou et al. 1992; Garcia et al. 1992).

The complete amino acid sequence of the human progesterone receptor has been deduced from cloned cDNA by Misrani et al. (1987).

Structural requirements of the ligand and mapping of the hormone-binding site of the progestin receptor have been discussed by Ojasoo and Raynaud (1990).

Allan et al. (1992) studied conformational changes in the ligand binding domain induced by various progestins and antiprogestins.

Collins (1994) recommended the ratio between the affinity of a compound to progesterone receptors to the affinity to androgen receptors as a selection criterion for new oral contraceptives.

Comparative pharmacology of newer progestagens has been reviewed by Kuhl (1996).

Oñate et al. (1994) found that the DNA-binding protein HMG-1 enhances progesterone receptor binding to its target DNA sequences.

The concept of two categories of progestin antagonists based on differences how the interact with and inactivate the progestin receptor has been discussed by Edwards et al. (1995).

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N.3.2.1.2 Transactivation assay for gestagens

PURPOSE AND RATIONALE

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor acts with hormone responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon (1988).

Transactivation assays were used by several authors to test the progestional and antiprogestional activity of steroidal and non-steroidal compounds (Pathirana et al. 1995; Sobek et al. 1994; Jones et al. 1996; Dijkema et al. 1988; Edwards et al. 1998; Schoonen et al. 1998; Zhi et al. 1998)

PROCEDURE

CV-1 cells (African green monkey kidney fibroblasts) are grown in Dulbecco's modified Eagle medium containing 10% charcoal resin-stripped fetal bovine se-

rum, 2 mM glutamine, and 55 µg/ml gentamycin. Cells are maintained in an environment of 4% carbon dioxide and routinely passaged from T-255 flasks to 96-well microtiter plates (1.5×10^5 cells/well, 70% confluent) 1 day before transfection.

Cells are transiently transfected, by the standard calcium phosphate co-precipitation procedure with 50 ng/well of plasmids coding for: hPR(human progesterone receptor)-B (hPR-B1) and pRS-β-Gal under constitutive control and a reporter (MTV-LUC) containing a response element for the progesterone receptor. After 6 h, the medium is replaced with medium containing progesterone at concentrations between 10^{-11} and 10^{-5} M or another progestagen. After a 40 h incubation, wells are washed with phosphate buffered saline and the cells are lysed with Triton X-100-based buffer. An aliquot of lysate (20 µl) is then transferred to Dynatech 96-well plates containing 1.6 mM ATP. LUC activity (chemiluminescence upon addition of luciferin substrate) is determined using a Dynatech ML1 000 luminometer, according to the equation:

$$\text{LUC units} = \text{relative LUC units} \times 10^4$$

β-Gal activity is determined from the remaining lysate in the original 96-well plates. The substrate, o-nitro-phenol-β-galactoside, is added to the plates, followed by incubation at 37 °C. The incubation is terminated by addition of sodium carbonate when the average absorbance, as determined by visual observation of the yellow product (o-nitrophenol), is within a standard range. Absorbance at a wavelength of 415 nm is then quantified spectrophotometrically. β-Gal rates are calculated according to the following equation:

$$\beta\text{-Gal rate} = \frac{\beta\text{-Gal absorbance} \times 10^5}{\beta\text{-Gal incubation time}}$$

For each set of replicate wells, normalized response is calculated according the following equation:

$$\text{Normalized response} = \text{LUC units} / \beta\text{-Gal rate}$$

EVALUATION

Agonist activity is determined by examining the amount of LUC expression (normalized response). The effective concentration that produces 50% of the maximal response (EC_{50}) is quantified. The efficacy is a function of the LUC expression relative to the maximal LUC expression produced by the reference agonist, e.g., progesterone. Antagonist activity is determined by testing the amount of LUC expression in the presence of a fixed concentration (equal to its agonist EC_{50}) of reference agonist. The concentration of test compound that inhibits by 50% the gene expression induced by the reference compound is quantified (IC_{50}). In addition, the efficacy of antagonists is determined as a function of maximal inhibition (LUC expression = basal activity).

CRITICAL ASSESSMENT OF THE METHOD

See N.2.1.1.2.

MODIFICATIONS OF THE METHOD

For transactivation studies, hPRA-A-MMTV-LUC and hPR-B-MMTV-LUC and stably co-transfected CHO cells were used by Dijekema et al. (1998), Schoonen et al. (1998).

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N.3.2.1.3 Alkaline phosphatase assay

PURPOSE AND RATIONALE

Progestins induce the *de novo* synthesis of alkaline phosphatase (Di Lorenzo et al. 1991, 1993). The progestin in-duction of the nonspecific tissue alkaline phosphatase is not altered by other steroid hormones or synthetic analogs. This finding has been used by Sobek et al. (1994), Pathirana et al. (1995), Li et al. (1997) to measure the progestogenic and antiprogestogenic actions in a microplate assay.

PROCEDURE

T47D cells are grown in 96-well plates near confluence and then treated with 0.1–0.2% alcohol (control) or steroid agonists; otherwise R 5 020 plus potential antagonist for 2 days. The cells are then fixed with 3.7% formaldehyde in phosphate buffered saline for 15 min at 15 °C, washed with 200 µl phosphate buffered saline and stored at –90 °C. After thawing, each well is incubated with 100 µl *p*-nitrophenyl phosphate (1 mg pNPP/ml) in DEAM (1 M diethanolamine, pH 9.8, containing 0.5 mM $MgCl_2$, and 20 µM $ZnSO_4$). The enzyme reaction is allowed to proceed in the dark at 37 °C. Formation of *p*-nitrophenol is monitored periodically at 405 nm in a microplate reader. In each microtiter plate, blanks, *p*-nitrophenol, and alkaline phosphatase standards are measured together with the samples. One unit of alkaline phosphatase is defined as the amount of enzyme capable of transforming 1 µmole of substrate in 1 min at 37 °C. Enzyme assays are performed under conditions of linearity relative to the substrate and to the concentration of proteins.

EVALUATION

Progestin concentrations corresponding to half the maximal increase in alkaline phosphatase activity (ED_{50}) are calculated graphically and by computer assisted analysis using the ImmunoFit EIA/RIA analysis software (Beckman Instruments, Inc.).

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N.3.2.2 *In vivo* methods

N.3.2.2.1 Clauberg (McPhail) test in rabbits

PURPOSE AND RATIONALE

For this historical bioassay, Clauberg (1930a–d) first described the histological changes of the endometrium in estrogen pretreated rabbits after administration of progestional compounds. This test was further studied by Butenandt et al. (1934) and systematically examined by McPhail (1934) who introduced scores for the changes of the endometrium.

PROCEDURE

Immature female rabbits (Yellow Silver or New Zealand strain), weighing 550–650 g, receive daily injections of 5.0 µg estradiol benzoate per animal in sesame oil solution for a period of 6 days (priming). From the 7th until the 12th day (treatment for 5 days) the rabbits are administered the test drug or the standard in several doses. 3 to 4 animals are used for each dosage group. Standard doses are: 0.02, 0.08 and 2.0 mg progesterone per day and animal s.c. in sesame oil solution, or 0.01, 0.02, and 0.04 mg medroxyprogesterone acetate orally. Controls receive either the vehicles or estradiol benzoate only. On the 15th day, the animals are sacrificed, both horns of the uterus removed and fixed in 10% formalin. Sections are made from the middle part of each horn for histological examination.

EVALUATION

Rabbits treated with estradiol benzoate have an increased uterine weight (priming), progestagens induce further proliferation and secretory transformation. The following scores are established:

0 ramification of the uterus mucosa, but no proliferation (estrogen treatment only)
1 slight proliferation of the uterus mucosa
2 medium proliferation of the uterus mucosa, slight additional ramification
3 pronounced proliferation of the uterus mucosa
4 very pronounced proliferation of the uterus mucosa, pronounced ramification.

The scores from each dosage group are averaged. Dose-response curves are constructed in order to calculate potency ratios versus the standard (progesterone).

The evaluation needs some experience of the investigator. Preliminary experiments with standard drugs and photographical documentation of the histological findings are recommended.

MODIFICATIONS OF THE METHOD

In order to study the prolonged activity, the animals are pretreated with estradiol for 6 days, followed by a single subcutaneous injection of the test compound or hydroxyprogesterone capronate as standard. Daily treatment with estradiol is continued and the rabbits sacrificed at various intervals up to 4 weeks.

McGinty et al. (1939) established a local **progestional test** which involves the direct injection of progesterone into an uterine segment. The test is performed in immature rabbits primed for 6 days with estrogen. On the 7th day, the uterus is exposed by lapa-rotomy. The upper middle segment of each horn is ligated without disturbance of blood circulation. A solution of the gestagen in oil is injected into the lumen of one segment through the lower ligature, which is drawn tight after the injection. In the opposite horn, only the vehicle is injected. Three days later, the animals are sacrificed and sections of the horn are evaluated histologically according to the McPhail scores (Tayama et al. 1979).

Pincus et al. (1957) improved the quantitative aspects of the McPhail test with the planimetric measurement of the endometrial proliferation.

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N.3.2.2.2 Endometrial carbonic anhydrase

PURPOSE AND RATIONALE

Carbonic anhydrase activity in the endometrium is increased after administration of progesterone. Carbonic anhydrase activity in the endometrium of rabbits is used for a quantitative progestin assay (Lutwak-Mann 1955; Miyake and Pincus 1958).

PROCEDURE

Immature female rabbits are estrogen primed and progestin treated as described in the Clauberg-test. After sacrifice of the animals, the uteri are opened longitudinally. Endometrium is dissected, weighed and homogenized with 10-fold volume in a glass homogenizer. After centrifugation, carbonic anhydrase is determined in the supernatant with the colorimetric method of Philpot and Philpot (1936) using bromo-thymol as indicator.

EVALUATION

Mean values of carbonic anhydrase activity/g wet tissue are calculated and potency ratios for test compound and standard established.

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N.3.2.2.3 Deciduoma formation

PURPOSE AND RATIONALE

This is a classical bioassay for progestagens. The deciduoma tests are based on the fact that the endometrium of the estrogen-primed, progesterone-treated rodent is sensitive to local stimuli such as scratching (Astwood 1939), chemical irritation, and electrical stimulation, and produces a deciduoma, as the equivalent of a maternal placental tumor.

PROCEDURE

Adult female Sprague-Dawley rats weighing 200 to 250 g are ovariectomized and 1 week later treated with 0.5 μg estradiol/animal once daily subcutaneously for 4 days, followed by 9 days of progesterone or the test compound in various doses. The uterus is exposed on the fifth day of progesterone treatment and 1.0 mg histamine dihydrochloride is injected into the lumen of one horn. The animals are sacrificed after the last treatment. Both uterine horns are removed and weighed. The degree of deciduoma formation is evaluated by the percent increase in the weight of the histamine-injected uterine horn as compared with the control horn.

EVALUATION

Dose-response curves are established plotting % increase in weight of the treated uterus horn versus logarithm of dose of test compound and standard in order to calculate potency ratios.

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N.3.2.2.4 Pregnancy maintenance

PURPOSE AND RATIONALE

This is a historical bioassay for progestational activity. In the rat, ovariectomy performed during the first half of pregnancy terminates gestation, but when performed during the second half of pregnancy does not result in abortion, because of the capacity of the placenta to produce progestin and estrogen. Maintenance of pregnancy after ovariectomy in early pregnancy can be achieved by sufficient exogenous progestin with and without estrogen.

PROCEDURE

Mature Sprague-Dawley female rats are inseminated by being placed with males overnight. The day that sperms are found, is considered to be day one of pregnancy. On day 8, the females are ovariectomized if found pregnant upon examination of the uterus. Test compounds are administered once daily, subcutaneously, for 13 days beginning immediately after ovariectomy. Estradiol (0.1 μg/day) is administered concomitantly with the test compound. On day 21, the animals are sacrificed and autopsied. Presence or absence of implantation sites, and the numbers of live embryos are recorded.

EVALUATION

Normal pregnant rats have an average of 11 implantation sites and about 10 live embryos. A net success index is calculated as percentage of this theoretical maximum effect of pregnancy maintenance.

MODIFICATIONS OF THE METHOD

Similar tests have been performed in rabbits (Elton and Edgren 1958), in mice (McGinty 1959), and in hamsters (Shipley 1965). In rabbits, abortion can be induced by intravenous injection of oxytocin from day 30 of gravidity onwards. This effect can be suppressed by potent gestagens.

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N.3.2.3
Anti-progestional activity

N.3.2.3.1
Progesterone antagonism

PURPOSE AND RATIONALE

The anti-progestional activity of a test compound may be determined by antagonism against the effect of progesterone in the Clauberg/McPhail assay in rabbits (N.3.2.2.1) or the McGinty test (N.3.2.2.1) and in the deciduoma formation assay in rats (N.3.2.2.3).

MODIFICATIONS OFTHE METHOD

Progesterone control of cervical ripening in the guinea pig and the tree shrew *Tupaja belangeri* has been used for the evaluation of progesterone antagonists (Chwalisz et al. 1991; Chwalisz 1994). The effects of progesterone antagonists on surgically induced endometriosis in rats have been studied by Stöckemann and Chwalisz (1993).

Michna et al. (1991) developed a bioassay which allows quantification of the antiproliferative potency of progesterone antagonists on the mammary gland in rats. Female Wistar rats with a body weight of 100 g were ovarectomized under anesthesia. One week after ovariectomy the rats were substituted with 10 µg estrone and 3 mg progesterone for 3 days. The animals in the experimental groups simultaneously received the progesterone antagonist. The animals were sacrificed and the inguinal mammary glands dissected: the right gland for biochemical analysis (DNA), the left for morphometrical analysis. The entire inguinal mammary gland was prepared for conventional paraffin sections and stained with ferric ammonium sulfate. Microphotographs were taken in transmitted light. The number of tubulo-alveolar buds was counted in the whole mount preparations using a 40-fold magnification. In the neighborhood of the inguinal node, a square of 2.5 mm^2 was quantified and calculated for a tissue volume of 100 mm^3 in more than 10 animals. The antiproliferative action of progesterone antagonists on the amount of tubulo-alveolar buds was estimated for a 80% confidence interval of mean inhibition.

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N.3.2.3.2 Luteolytic effect of prostaglandins

PURPOSE AND RATIONALE

Synthetic prostaglandins are used for synchronization of estrus and for treatment of anestrus caused by a persistent corpus luteum in cattle. Furthermore, synthetic prostaglandins were studied for termination of early pregnancy in humans (Karim et al. 1977; Takagi et al. 1977, 1978; Toppozada et al. 1979). The most useful animal model for luteolytic activity is the hamster (Gutknecht et al. 1971; Labhsetwar 1971, 1972a; Dukes et al. 1974; Bartmann et al. 1979; Galliani et al. 1984; Roy et al. 1987). Other species such as rat (Fuchs et al. 1974), mouse (Labhsetwar 1972b), and guinea pig (Blatchley and Donovan 1969) have also been studied.

PROCEDURE

Adult female golden hamsters (*Mesocricetus auratus*), weighing approximately 100 g with regular, 4-day estrus cycles, are housed under controlled light and temperature conditions and given a standard diet. They are caged with fertile males on the day before expected vaginal discharge and the next morning vaginal smears are taken. If clumps of spermatozoa are found in the smear, this day is designated as day 1 of pregnancy. Groups of 10 animals are treated on days 4, 5, and 6 with four different doses of standard (prostaglandin F2α) or test compound. On day 13 of pregnancy, the hamsters are sacrificed and tested for pregnancy by counting the number of implantation scars.

EVALUATION

The luteolytic activity is expressed as the median effective dose, at this dose pregnancy can be terminated in 50% of the treated animals (ED_{50}).

MODIFICATIONS OF THE METHOD

The mode of action of luteolysis by prostaglandins has been studied by various authors both *in vitro* (Speroff and Ramwell 1970; O'Grady et al. 1972; Henderson and McNatty 1975; Kenny and Robinson 1986; Brambaifa 1988) and *in vivo* (Pharriss and Wyngarden 1969; Johnston and Hunter 1970; McCracken et al. 1970; Chatterjee 1973; Buhr et al. 1983; Torjesen and Aakvaag 1984, 1986).

Cao and Chan (1993) investigated the effects of oxytocin and luteal prostaglandins on the functional regression of the corpus luteum in pseudopregnant rats.

Motta et al. (1996) studied the effect of an oxytocin receptor antagonist on ovarian and uterine synthesis and release of prostaglandin $F_{2\alpha}$ in pseudopregnant rats.

Stocco and Deis (1998) examined the participation of intraluteal progesterone and prostaglandin $F_{2\alpha}$ in LH-induced luteolysis in pregnant rats.

Luteolysis by prostaglandins in the rhesus monkey has been studied by Auletta and Kelm (1994) and Auletta et al. (1995).

The ewe as a model for regulation of luteal regression has been recommended by Hoyer (1998).

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N.4 Testicular steroid hormones

N.4.0.1 Castration of male rats

PROCEDURE

Castration of young male rats is performed with minimal bleeding in animals weighing less than 60 g. The animal is anesthetized. A small transversal incision is made in the skin on the ventral site over the symphysis. The testis lying in the scrotum is gently pushed into the abdominal cavity. With a pair of fine forceps, the abdominal wall is opened. The epididymal fat pad, easily to be seen, is grasped with the forceps and the testis with the epididymis is pulled out from the wound. The ductus deferens with the testicular vessels is crushed with an artery forceps and the testis together with the epididymal fad pad cut off with a pair of fine scissors. There is almost no bleeding in young animals. In older animals, ligation of the testicular vessels together with the ductus deferens may be necessary. The same procedure is performed on the other side. The skin wound is closed with one or two wound clips. The animal recovers immediately. With some skill, the operation can be performed very rapidly.

MODIFICATIONS OF THE METHODS

Dorfman (1969) recommended to remove the testes through an incision in the tip of the scrotum. In our hands, the procedure described above was preferable.

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N.4.0.2 Caponizing of cockerels

PROCEDURE

This is a classical bioassay for androgens. White Leghorn cockerels are used for surgery at approximately 6 weeks of age. The animals being fasted 24 h prior to surgery are anesthetized with ether and placed on their sides. An incision is made between the last two ribs, the muscle layer is divided, and the incision is pulled apart with small retractors. The testis is found close to the midline of the posterior abdominal wall, alongside

the vena cava. The capsule enclosing the testis is cut and the gonad is removed. It is imperative to remove the testis intact, as fragments left behind are usually vascularized and persist, giving rise to incompletely caponized animals. The incision is closed by suture. The second testis is removed in a similar fashion.

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N.4.1
Androgenic and anabolic activity

N.4.1.1
In vitro methods

N.4.1.1.1
Androgen receptor binding

PURPOSE AND RATIONALE

Rat ventral prostate (Bonne and Raynaud 1974; Liao et al. 1974; Grover and Odell 1975; Ojasoo and Raynaud 1978; Raynaud et al. 1979; Winnecker et al. 1989; Duc et al. 1995) or mouse kidney (Isomaa et al. 1982) serve as sources for androgen receptors. Moreover, human androgen receptors have been prepared from transfected COS-1 cells (Teutsch et al. 1994). As ligands, labeled androstanolone, 5α-dihydrotestosterone, testosterone, and, more recently, methyltrienolone (R 1 881) have been used.

PROCEDURE

Androgen receptor assay. Cytosol is prepared from ventral prostate glands of adult male rats castrated approximately 24 h before use. The tissue is homogenized in TMDG buffer (10 mM Tris, 20 mM sodium molybdate, 2 mM dithiothreitol, 10% glycerol, pH = 7.4) at room temperature using a motor driven glass homogenizer and centrifuged at 135 000 *g* for 1 h. Aliquots of the supernatant (cytosol) are diluted to contain 40 mg tissue/ml and incubated for 1 h or overnight with [17α-methyl-^{3}H]R 1 881 (methyltrienolone, 5 nM final concentration, 87 Ci/mmole, New England Nuclear) in either the absence or presence of increasing concentrations (1 nM–10 μM) of R 1 881 or test compounds. Because R 1 881 binds weakly to progesterone and glucocorticoid receptors, cytosols are pretreated with 1 μM triamcinolone acetonide to block these interactions. After 1 or 18 h incubation period, a suspension of dextran-coated charcoal (1% charcoal, 0.05% dextran T-70, 0.05% BSA) is added to the ligand-cytosol mixture and incubated for 5 min. The charcoal is removed by centrifugation at 1 500 *g* for 10 min and the supernatant (protein-bound [^{3}H]R 1 881) counted using 10 ml of Biofluor liquid scintillation fluid (New England Nuclear) in a liquid scintillation spectrometer.

Nuclear androgen receptor exchange assay. Ventral prostates are homogenized at 100 mg/ml in hexylene glycol buffer (1 M hexylene glycol, 1 mM $MgCl_2$, 2.0 mM dithiothreitol, 5.0 mM EGTA, 1.0 mM PIPES, pH = 7.4) using a motor driven ground glass homogenizer. Homogenates are centrifuged at 1 500 *g* for 10 min. The nuclear pellet is washed 3 times in homogenization buffer by gently resuspending the pellet in a Dounce homogenizer and centrifugation at 1 500 *g* for 10 min. The washed nuclear pellet is resuspended in pyridoxal-5'-phosphate extraction buffer (20 mM sodium barbital, 5 mM pyridoxal-5'-phosphate, 5.0 mM dithiothreitol, 1.5 mM EDTA, 150 mM KCl, 20% glycerol, pH = 7.4) for 60 min at a final concentration of 60 mg tissue/ml. The extracted nuclei are centrifuged at 25 000 *g* for 30 min with the resulting supernatant being used in the same single saturating dose assay as described for prostate cytosol.

EVALUATION

The binding of test substances to the androgen receptor is quantified by calculating relative binding affinity (ratio of the molar concentration of unlabeled R 1 881 to test substance required to inhibit the binding of [^{3}H]R 1 881 by 50% after correction for non-specific binding) and equilibrium inhibitory binding constant ($K_i = IC_{50}/(1 + C / K_d)$, where C = the concentration of [^{3}H]R 1 881 and the K_d for R 1 881 is 1.3 nM.

In the nuclear androgen receptor exchange assay treatment group means are compared to control means, using ANOVA and Dunnett's multiple comparison tests.

MODIFICATIONS OF THE METHOD

Cells assays and animal assays were described.

Brown et al. (1981) studied anti-androgen effects on androgen receptor binding in cultured human newborn foreskin fibroblasts.

Tezón et al. (1982) studied the intracellular distribution of the androgen receptor in the rat epididymis under the influence of androgens and anti-androgens.

The use of tritiated 7α,17α-dimethyl-19-nortestosterone for the assay of androgen receptors was recommended by Schilling and Liao (1984).

Characterization and expression of a cDNA encoding the human androgen receptor was described by Tilley et al. (1989).

Hoyte et al. (1993) recommended 7a-methyl-17α-(E-2'-[^{125}I]iodovinyl)-19-nortestosterone as radioligand for the detection of the androgen receptor.

Structure-affinity relationships of various steroids structurally related to nomegestrol and progesterone for [3H]testosterone binding to rat ventral prostate cytosol were reported by Botella et al. (1987).

Molecular cloning of human androgen receptor complementary cDNA has been reported by Chang et al. (1988), and Lubahn et al. (1988).

DNA-binding of androgen receptor overexpressed in COS-1 cells has been reported by von Krempelhuber et al. (1994).

Thoth et al. (1995) studied *in vitro* binding of 16-methylated C-18 and C-19 steroid derivates to the androgen receptor using cytosol of castrated rat prostate and [^{3}H]R 1 881 as radioligand.

Chang et al. (1995) reviewed the structure and function of the androgen receptor and its role playing in the function of the mammalian system.

The interaction of androgen receptors with the androgen response element in intact cells was investigated by Karvonen et al. (1997).

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N.4.1.1.2 Transactivation assay for androgens

PURPOSE AND RATIONALE

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor acts with hormone responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon (1988). The hormone-dependent transcriptional activation can be determined in tissue culture by transfection of the steroid receptor under investigation and a reporter gene linked to a hormonally responsive promotor into cells. The transactivation assay allows determination of the agonistic and also the antagonistic potency of a given compound, by either induction or inhibition of reporter gene activity (Fuhrmann et al. 1992).

PROCEDURE

Vector construct

CV-1 cells are stably transfected with the rat androgen receptor and pMMTV-CAT7. The pMMTV-CAT plasmid containing the mouse mammary tumor virus promotor linked to a chloramphenicol acetyltransferase gene is prepared according to Cato et al. (1986).

Cell culture and transfections

The culture medium of CV-1 cells stably transfected with the rat androgen receptor and pMMTV-CAT7 is supplemented with 400 µg/ml G418 (Gibco BRL) and 5 µg/ml puromycin.

Stable and transient transfections are performed using Lipofectin reagent (Gibco BRL) according to a procedure recommended by Felgner and Holm (1989). Stable transfections are carried out according to Fuhrmann et al. (1992). For transient transfection, 1×10^6 COS-1 or CV-1 cells, respectively, are plated onto 100-mm dishes one day prior to transfection. Cells are typically about 80% confluent after 24 h. Before transfection, cells are washed twice with 1 ml Opti-MEM (Gibco BRL) per dish. For each dish, 5 µg hAR expression plasmid and 5 µg pMMTV-CAT are diluted with 1 ml Opti-MEM; in addition, 50 µg Lipofectin Reagent is diluted with 1 ml Opti-MEM. Next, DNA and Lipofectin Reagent dilutions are combined in a polystyrene snap-cup tube to obtain 2 ml of transfection solution per dish, gently mixed, incubated at room temperature for 5 min, and added to the washed cells. After 5 h the transfection solution is replaced by 6 ml DMEM supplemented with 10% fetal calf serum.

To study the effect of the test hormones, transiently transfected cells are trypsinized, pooled and replated onto 60-mm dishes at a density of 4.5×10^5 per dish 24 h after transfection. Stably transfected cells are seeded onto 6-well dishes (1×10^5 cells per dish). Cells are cultured in medium supplemented with 3% charcoal-stripped fetal calf serum and the appropriate hormones for 48 h. As negative control for the reporter gene induction, cells are cultured with 1% ethanol. Transactivation assays with transiently or stably transfected cells are carried out at least three times.

CAT assay

Transiently transfected cells and stably transfected cells are disrupted by freezing (ethanol/dry ice bath) and thawing (37 °C water bath) three times. Protein concentrations of the cell extract are determined according to Bradford (1976). The CAT assay is performed according to Gorman et al. (1982).

EVALUATION

CAT activity is calculated as percent conversion from chloramphenicol to acetylated chloramphenicol. Concentration-response curves for CAT induction are established to demonstrate the potency of the test hormone. The synthetic androgen R 1 881 (10^{-10} to 10^{-6} mol/l) serves as standard.

For antiandrogenic activity, CAT activity in the presence of 0.5 nmol/l R 1 881 is set as 100% and relative CAT activity is calculated as percentage of this value. Concentration-response curves for CAT inhibition are established with increasing concentrations of the antihormone.

CRITICAL ASSESSMENT OF THE METHOD

See N.2.1.1.2.

MODIFICATIONS OF THE METHOD

Warriar et al. (1993) examined the ability of dehydrotestosterone and various antiandrogens to stimulate or to inhibit the transcription activation of mouse mam-

mary tumor virus-bacterial chloramphenicol acetyltransferase (MMTV-CAT) in CV-1 cells.

White et al. (1994) described a simple and sensitive high-throughput assay which can be adapted for several classes of steroid agonists and antagonists. A DNA cassette, containing a synthetic steroid-inducible promotor controlling the expression of a bacterial chloramphenicol acetyltransferase gene (GRE5-CAT), was inserted into an Epstein-Barr virus episomal vector which replicates autonomously in primate and human cells. This promotor/reporter system was used to generate two stably transfected human cell lines. In the cervical carcinoma cell line HeLa, which expresses high level of glucocorticoid receptor, the GRE5 promotor is inducible over 100-fold by the synthetic glucocorticoid dexamethasone. In the breast carcinoma cell line T47D, which expresses progesterone and androgen receptors, the GRE5 promotor is inducible over 100-fold by either progesterone or dihydrotestosterone. These cell lines can be used to screen large numbers of natural and synthetic steroid agonists and antagonists in microtiter wells directly using a colorimetric chloramphenicol acetyltransferase assay.

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N.4.1.2 *In vivo* methods

N.4.1.2.1 Chicken comb method

PURPOSE AND RATIONALE

This classical bioassay based on growth of the capon comb has been used by many authors for androgenic activity and found to be extremely useful for the isolation and structural elucidation of natural androgens. Many modifications have been published (Dorfman 1969).

PROCEDURE

Prior to assay, the surface area (sum of the length plus height of each individual comb) is determined by a millimeter rule placed directly on the comb. The capons are injected daily intramuscularly for 5 consecutive days with a solution or suspension of the test compound or the standard in 1 ml olive oil. Twenty-four hours after the last injection, the combs are re-measured and the growth of the comb is expressed as the sum of length and height in millimeters. Groups of 8 animals are used for at least 2 doses of the test compound and the standard.

EVALUATION

The mean values of each group are calculated and plotted as dose-response curve for the test compound and the standard in order to calculate potency ratios.

MODIFICATIONS OF THE METHOD

The hormones, dissolved in oil, have been applied locally to the capon's comb. A greater sensitivity has been achieved with this modification (Fussgänger 1934; McCullagh and Cuyler 1939).

Newly hatched chicks of either sex have been used to study the growth of combs after systemic or local administration (Frank et al. 1942; Dorfman 1948). White Leghorn chicks are used at an age of 2–3 days. They are kept in a brooder with a thermostatic control. An oily solution (0.05 ml) of the test compound or the standard is applied on the comb daily for a period of 7 days. Twenty-four hours after the last application, the animals are autopsied. Body weights are determined. The combs are removed by two longitudinal incisions along the base of the comb at its juncture with the scalp. The comb is freed from the scalp, touched lightly on a towel to remove blood and weighed. Dose-response curves are established.

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N.4.1.2.2 Weight of ventral prostate, seminal vesicles and musculus levator ani

PURPOSE AND RATIONALE
Androgens affect the development of secondary sex organs in the male. In the rat, the growth of the ventral prostate, the seminal vesicles and the musculus levator ani is dependent on the presence of male sexual hormones. Weight increase of the musculus levator ani is considered to indicate anabolic activity.

PROCEDURE
Immature male Sprague-Dawley rats weighing about 55 g are orchidectomized as described above. The animals are administered the test compound in various doses orally in 0.5 ml 0.5% carboxymethylcellulose or subcutaneously in 0.2 ml sesame oil suspension daily over a period of 10 days. Testosterone is given in doses of 0.02, 0.1, and 0.5 mg per animal subcutaneously or methyltestosterone in doses of 0.25, 1.5, and 5 mg per animal serve as standards. Controls receive the vehicle only. Ten animals are used for each group. On the 11^{th} day, the animals are sacrificed and the seminal vesicles, the ventral prostate, and the musculus levator ani carefully dissected and weighed. The seminal vesicles are squeezed between filter paper to remove the fluid.

Dissection of the levator ani muscle is performed after removal of the skin in the scrotal area between the base of the penis and the anus. The posterior aspect of the perineal complex is cleared of fat and connective tissue with forceps, particular care being taken to expose the constrictions at either end of the levator ani where it joins the bulbocavernosus muscle. The rectum is transected just caudad to the point where the musculus levator ani loops around it dorsally. The body of the levator ani is then freed of the rectum and is removed by incisions at the points of attachment to the bulbocavernosus muscle. The levator ani is cleared of any connective tissue and weighed to the nearest 0.1 mg. Body weight of the animals is registered at the beginning and at the end of the experiment.

EVALUATION
The ratio organ weight/body weight is calculated for each organ and for each animal (relative organ weights). Dose-response curves are constructed for each organ comparing the test compound with the standard in order to calculate potency ratios. Increase in weight of seminal vesicles and ventral prostate indicates androgenic activity, whereas increase in weight of musculus levator ani is considered to indicates anabolic activity. In evaluating steroids for possible use as anabolic agents, Hershberger et al. (1953) suggested the use of the levator ani: ventral prostate ratio, which is defined as the ratio of the increase in levator ani weight divided by the increase in ventral prostate weight.

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N.4.1.2.3 Nitrogen retention

PURPOSE AND RATIONALE
Anabolic agents induce positive nitrogen balance in the living organism. Many modifications of this assay

principle have evolved. Stafford et al. (1954) suggested a method involving the measurement of nitrogen excretion in the castrated rat fed a liquid diet and in nitrogen balance.

PROCEDURE

Twenty-five day old rats are castrated and kept untreated for 67 days, reaching about 300 g in body weight on normal laboratory diet. After 67 days, they are changed to a liquid diet-force feeding regime. Besides carbohydrates and fat, the diet contains casein and brewer's yeast as nitrogen source. At the start, the rats receive 10 ml per day, and this is increased to 26 ml per day. This feeding is continued for 30 days with simultaneous administration of the test drug once a day. Twenty-four hour urine specimens are collected 3 times weekly and analyzed for total nitrogen.

EVALUATION

Indices are calculated, such as greatest daily retention which is defined as the difference between the lowest daily nitrogen value after beginning of treatment and the pre-injection mean, the total nitrogen retention which is the sum of the differences between the pre-injection excretion and the daily values during the retention period, and the number of days in the retention period.

MODIFICATIONS OF THE METHOD

A method for the assay of anabolic steroids in the monkey (*Macaca mulatta*) has been suggested by Stucki et al. (1960). Nitrogen retention expressed as total nitrogen retained per day during the treatment period is chosen as end point.

CRITICAL ASSESSMENT OF THE METHOD

The assays can be used for demonstration of the anabolic effect but are cumbersome for quantitative comparisons test compounds and standard.

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N.4.2
Anti-androgenic activity

N.4.2.0.1
General considerations

Anti-androgens exhibit their activity both on a peripheral and a central site (Neumann et al. 1970; Mainwaring 1977; Neri 1977; Neumann et al. 1977; Raynaud et al. 1977; Neumann 1985; Moguilewski and Bouton 1988). They compete with the peripheral androgen receptors and thus inhibit the effect of endogenous or exogenous androgens. They inhibit gonadotropin secretion and thus diminish testosterone production by the gonads. In addition to their effects on reproduction and accessory sexual organs, anti-androgens are tested for inhibition of sebum production as potential anti-acne drugs. The methods for gonadotropin inhibition are described in Sect. N.7.1.8.

Inhibition of 5α-reductase, an enzyme located in tissues such as the prostate, where 5α-dihydrotestosterone is the specific androgenic hormone, is one mechanism to inhibit benign prostate hyperplasia in men.

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N.4.2.1
In vitro methods

N.4.2.1.1
Inhibition of 5α-reductase

PURPOSE AND RATIONALE

Testosterone is converted to 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase which is specifi-

cally localized in some androgen-target tissues, such as prostate, seminal vesicle, epididymis and skin, whereas in other androgen-sensitive tissues, such as the skeletal muscles and the central structures, the androgenic stimulus is mediated by testosterone itself. Inhibition of 5α-reductase provides a selective approach to androgen deprivation in DHT-target tissues, such as the prostate. Therefore, 5α-reductase inhibitors are proposed for therapy of benign hyperplasia of the prostate.

PROCEDURE

5α-Reductase can be prepared from prostates of various species, such as human, dog, and rat.

Frozen human prostates obtained from benign prostatic hyperplasia patients are thawed and minced with a pair of scissors into small pieces. The minced tissue is homogenized in 3 tissue volumes of medium A (20 mM potassium phosphate, pH 6.5, containing 0.32 M sucrose, 1 mM dithiothreitol and 50 µM NADPH) with a Brinkmann Polytron and a glass-glass homogenizer. The homogenate is centrifuged at 140 000 *g* for 60 min and the pellets are washed with approximately 3 tissue volumes of medium A. The washed pellets are suspended at a concentration of 5–10 mg protein/ml in 20 mM potassium phosphate, pH 6.5, containing 20% glycerol and 1 mM dithiothreitol.

Dog prostatic particulates are prepared from either fresh or frozen prostates from male mature mongrel dogs as described for human prostate. The washed pellets are suspended in medium A at a concentration of 30–60 mg protein/ml.

Ventral prostates from male Sprague-Dawley rats weighing 400 g are processed as described for human prostate, except that medium A without NADPH is used throughout the procedures. NADPH prevents inactivation of human and dog 5α-reductases during the preparation, but the rat enzyme is stable without the coenzyme.

For the 5α-reductase assay, reaction solutions are prepared in duplicate tubes containing 1 µM [^{14}C]testosterone, 1 mM dithiothreitol, 40 mM buffer (potassium phosphate, pH 6.5, for the rat and for the dog enzymes; Tris-citrate, pH 5.0, for the human enzyme), prostatic particulate (1 mg protein), and NADPH (50 µM for reaction with rat enzyme, 500 µM for reaction with human and dog enzyme) in a final volume of 0.5 ml. Test compounds or standard as inhibitors are added in 5 µl ethanol at concentrations between 10^{-9}–10^{-5} M. The control tubes receive the same volume ethanol. The reactions for the rat and dog enzymes are started by the addition of the prostatic particulates. The human prostatic particulate is premixed with NADPH before starting the reaction. The reactions are linear for at least 1 h at 37 °C. The reactions are carried out for 10–30 min and are stopped with 2 ml ethyl acetate containing testosterone, 5α-dihydrotestosterone, and andostandione (10 µg each). After centrifugation at 1 000 *g* for 5 min, the ethyl acetate phase is transferred to a tube and evaporated under nitrogen to dryness. The steroids are taken up in 50 µl ethyl acetate. The solutions are applied to Whatman LK5DF silica plates and the plates are developed in either ethyl acetate-cyclohexane (1 : 1) at 25 °C or chloroform-methanol (96 : 4) at 4 °C. The plates are air-dried and the chromatography is repeated. Nonradioactive steroid standards are located by UV and by spraying with 1% $CeSO_4$/10% H_2SO_4 solution followed by heating. The radioactivity profiles are determined by scanning the plates or by scraping the silica in sections and counting in a scintillation counter. 5α-Dihydrotestosterone is the only radioactive product for the rat and human enzymes. With the dog enzyme 5α-dihydrotestosterone, 3α,17β-androstanediol, androstan-3,17-dione, and androstenedione are formed. The radioactivities of the first 3 products are combined for the calculation of the 5α-reductase activity.

EVALUATION

IC_{50} values are calculated based on at least 5 dilutions of test preparations or standard.

MODIFICATIONS OF THE METHOD

Using human genital skin fibroblasts and simian COS cells, specific inhibition of 5α-reductase type 1 has been observed (Hirsch et al. 1993).

Wennbo et al. (1997) reported that **transgenic mice** overexpressing the prolactin gene develop dramatic enlargement of the prostate gland.

Sigimura et al. (1994) described age-related changes of the prostate gland in the senescence-accelerated mouse and recommended this strain as a model of age-related changes in the prostate gland.

Neubauer et al. (1993) measured prostatic 5α-reductase in rats both *in vitro* and *ex vivo* and determined *in vivo* uptake of [^{3}H]testosterone by the prostate.

At least 2 isoforms of 5α-reductase have been isolated (Andersson and Russell 1990; Jenkins et al. 1992). Recombinant human prostatic 5α-reductase type I and II were expressed using the baculovirus-directed insect cell expression system (Iehlè et al. 1993).

Iehlè et al. (1995), di Salle et al. (1998) tested synthetic 5α-reductase inhibitors against both isoforms.

Tolman et al. (1995) identified a 4-azasteroid as a scalp isoenzyme selective inhibitor.

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N.4.2.2 *In vivo* methods

N.4.2.2.1 Chick comb method

PURPOSE AND RATIONALE

This description is purely of historical interest. Several modifications of the chick comb method were described for the androgen as well as the anti-androgen applied either systemically or locally.

PROCEDURE

One- to 3-day old male or female White Leghorn chicks are housed at constant temperature in a heated incubator. Testosterone is incorporated into the finely ground chick starting mash at a concentration of 80 mg per kilogram of food. The chicks are placed on this diet on day one. The test compound is dissolved in sesame oil. Each day for 4 days 0.1 ml of the oil solution is injected subcutaneously. Control chicks receive only the vehicle. Groups of 10 chicks are treated with various doses of the test compound or the standard. Twenty-four hours after the last injection, the animals are sacrificed, the combs removed and, after blotting of the cut edge, weighed rapidly to the nearest 0.5 mg. Body weights are also determined.

EVALUATION

The results are either expressed as absolute comb weights or as mg of comb per g of body weight. Results of groups treated with various doses of the inhibitor are statistically compared with controls receiving the vehicle only.

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N.4.2.2.2
Antagonism of the effect of testosterone on weight of ventral prostate, seminal vesicles and musculus levator ani

PURPOSE AND RATIONALE
In this modification, anti-androgens are administered to inhibit testosterone-stimulation of androgen-dependent organs in castrated rats.

PROCEDURE
Male Sprague-Dawley rats weighing 50–70 g are castrated. Starting one day after surgery, the rats are injected once daily for 7 days with 0.15 mg testosterone propionate in 0.1 ml sesame oil (standard stimulus). The test compound (antiandrogen) is dissolved or suspended in sesame oil at various doses and injected subcutaneously daily at a separate site for 7 days. Six to 10 animals are used per group. On the 8th day, the animals are sacrificed and weights of ventral prostate, seminal vesicles and musculus levator ani as well as body weight are recorded.

EVALUATION
The organ weight/body weight ratios is (relative organ weights are determined. The inhibition by the anti-androgen as compared with the group receiving testosterone propionate alone is calculated. Dose-response curves may be plotted for each organ by the percent inhibition versus the ratio of agonist to antagonist.

MODIFICATIONS OF THE METHOD
Dorfman (1962) described an anti-androgen assay using the castrated mouse. Weights of prostate and seminal vesicles were determined after injection of the antiandrogen test compounds and simultaneous injections of 2 mg testosterone over a period of 7 days.

APPLICATIONS OF THE METHOD
The degree of anti-androgenic activity is an important parameter in evaluation of the pharmacological activity of H_2-receptor antagonists (Winters et al. 1979; Broulik 1980; Baba et al. 1981; Sivelle et al. 1982; Foldesy et al. 1985; Takeda et al. 1982; Neubauer et al. 1990).

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N.4.2.2.3 Anti-androgenic activity in female rats

PURPOSE AND RATIONALE

This is another historical bioassay. Neumann and Elger (1966) described a method for testing the anti-androgenic activity of compounds in immature female rats. The inhibition by the anti-androgen cyproterone of the trophic effect of testosterone on uterine and preputial growth was studied in intact as well as in castrated female rats.

PROCEDURE

Female Sprague-Dawley rats weighing 40–45 g are ovariectomized. One week later, the treatment is started for 12 days with daily subcutaneous injections of 0.3 mg testosterone propionate and several doses of the antagonist. Controls receive testosterone propionate only. On the 13th day, the animals are sacrificed and the uteri and preputial glands weighed. Weight increase of female accessory sexual organs due to testosterone is dose-dependently reduced by an anti-androgen. Similar results are found using intact immature female rats.

EVALUATION

Dose-response curves are established for increasing doses of the anti-androgen at a given dose of testosterone propionate or for increasing doses of testosterone propionate at a given dose of the anti-androgen.

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N.4.2.2.4 Intra-uterine feminizing/virilizing effect

PURPOSE AND RATIONALE

From clinical observations as well as from experimental data (Neumann and Junkmann 1963), it is well known that the external genitalia of female fetuses can be masculinized by tumors secreting endogenous androgens or by steroids with androgenic activity. This effect can be antagonized by an anti-androgen.

PROCEDURE

Adult female Sprague-Dawley rats are mated and the beginning of pregnancy is determined by vaginal smears. From day 16 to day 19 of pregnancy, the antiandrogens are administered in various doses subcutaneously in sesame oil. Testosterone propionate is used in doses between 1.0 and 10.0 mg as androgenic stimulus. The dams are sacrificed on the 20th day of pregnancy and the external genitalia of the female embryos examined. The sex of the embryos is recognized by the presence of ovaries und uterus. A dose of 10 mg testosterone propionate leads to total masculinization of female embryos with loss of female and appearance of male sex characteristics. The anogenital distance in female rat fetuses measured macroscopically and microscopically is dose-dependent increased by testosterone propionate. This characteristic androgen effect is diminished by an anti-androgen.

EVALUATION

The androgen-dependent decrease of the anogenital distance in female fetuses by various doses of the anti-androgen is expressed as percentage inhibition of the testosterone-induced virilization.

MODIFICATIONS OF THE METHOD

Feminization of male rats was induced by treatment of pregnant rats during the second half of gestation and of the newborn fetuses during week 1–3 post partum with an anti-androgen, e.g., cyproterone acetate (Neumann and Elger 1966; Nishino et al. 1988; review by Neumann 1994). Decrease of the anogenital distance of male fetuses of anti-androgen-treated rats is expressed as percentage inhibition relative to fetuses from untreated mothers.

In feminized male rats, nipples and associated glandular tissues develop after birth as in normal female rats (Neumann and Elger 1967).

Feminization of male rats treated *in utero* was also observed with nonsteroidal anti-androgens and a 5α-reductase inhibitor (Imperato-McGinley et al. 1992).

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N.4.2.2.5 Anti-androgenic activity on sebaceous glands

PURPOSE AND RATIONALE

Bioassays for topical antiandrogens are based on inhibition of sebum secretion. Sebum production is increased by endogenous or exogenous androgens in many species including humans. In the mouse (Lapière and Chèvremont 1953; Neumann and Elger 1966), the Mongolian gerbil (Mitchell 1965), and the golden hamster (Hamilton and Montagna 1950), the male sex hormone stimulates sebum production and sebaceous gland growth. Morphometric evaluation by light microscopy in the rat has shown that castration causes a large reduction in the volume of the glands (Sauter and Loud 1975). The administration of testosterone over several days produces an enlargement of the sebaceous glands. This effect is used for morphometric evaluation of topical anti-androgens.

The method is described in detail in Sect. P.9.1.

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N.4.2.2.6 Anti-androgenic activity in the hamster flank organ

PURPOSE AND RATIONALE

This is another bioassay preferably for topical antiandrogens. The flank organs of Syrian golden hamsters are located on each flank of the animal consisting mainly of sebaceous tissue. Like sebaceous glands in other species, these pigmented spots respond to androgens by an increase in size. This proliferation is inhibited by systemic or topical anti-androgens.

The method is described in detail in Sect. P.9.5.

N.4.2.2.7 Effect of 5α-reductase inhibitors on plasma and tissue steroid levels

PURPOSE AND RATIONALE

5α-reductase inhibitors change the ratio of plasma testosterone and dihydrotestosterone as well as the tissue concentrations particularly in the prostate tissue.

PROCEDURE

Treatment of animals

Male Sprague-Dawley rats are treated s.c. with the 5α-reductase inhibitor or vehicle beginning on postnatal day 3 until the age of 4 or 7 weeks. After sacrifice, blood is withdrawn for testosterone and DHT determinations (George et al. 1989). Moreover, intraprostatic concentrations of testosterone and DHT are determined as an index of antiproliferative activity (di Salle et al. 1993).

Radioimmunoassay for testosterone and dihydrotestosterone

Serum testosterone and dihydrotestosterone are measured by radioimmunoassay in serum or serum extracts using specific antisera without prior chromatography. Serum samples of 0.5 ml may beextracted with 2 ml of freshly purified, peroxide-free diethylether by shaking for 60 s on a Vortex mixer. The aqueous phase is frozen at –70 °C, the ether phase containing steroids is transferred to conical test tubes, and evaporated under a stream of dry nitrogen. The dry residue is redissolved in BSA/phosphate buffer (1% BSA = bovine serum albumin) for RIA. (1,2,6,7-^{3}H)-Testosterone or (1,2,6,7-^{3}H)-dihydrotestosterone and specific antisera are added and tubes incubated over a period of 24 h at + 4 °C under non-equilibrium conditions. Bound hormone and free hormone are separated by adsorption on dextran-coated charcoal. The activity of each sample is determined by beta-spectrometry

Commercially available RIA-kitscan be used with suitable validation.

EVALUATION

The hormone concentrations in the sample are calculated from a standard curve by a computer program (e.g. RIA-Calc, LKB), using appropriate control sera. The ratios of testosterone to DHT in rats treated with different doses of 5α-reductase inhibitors are compared with vehicle-treated intact control rats.

MODIFICATIONS OF THE METHOD

di Salle et al. (1998) measured prostatic concentrations of testosterone and 5α-dihydrotestosterone in rats by specific radioimmunoassays after treatment with a dual type I and II 5α-reductase inhibitor. Similar measurements of tissue T/DHT ratios have been performed in dogs, in the context of pituitary down-regulation of androgen secretion by LHRH agonists.

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N.5 Thyroid hormones

N.5.0.1 General considerations

The thyroid gland secretes two types of hormones: the **thyroid hormones**, i.e., **thyroxin** (T4), **triiodothyronine** (T3) subserving metabolic functions and neuronal development, and the **calcitropic hormone, calcitonin**.

The main biological effects of **T3** and **T4** are on growth and development (e.g., maturation of tadpoles), the calorigenic effect (increase of basic metabolic rate), cardiovascular function (increased sensitivity of the heart to catecholamines), and metabolic functions (lipid, carbohydrate and collagen metabolism). The primary feedback effect is inhibition of TSH-secretion. These effects can be used for testing thyroid hormone analogues and metabolites. The iodine uptake and utilization in the thyroid can be inhibited by antithyroid drugs.

Historical bioassays rely on morphogenesis and neuronal development in **amphibia**. Thyroid hormones induce premature metamorphosis in amphibian tadpoles. Since the first observation by Gudernatsch (1913a,b) this phenomenon has been studied by numerous workers with the purpose to adapt this response for the assay of thyroidal substances (Bomskov 1937). Within a short period of time, the treatment with thyroid hormones induces the transformation of the **tadpole** into a small frog with growth of limbs, lungs, and other terrestrial accoutrements and stimulates the synthesis of enzymes mediating morphogenesis and transformation.

The **axolotl** (*Amblystoma mexicanum* or *tigrinum*) has been used as test object to study metamorphosis induced by thyroid hormones. This animal looses the gills and forms lungs, changing the shape of the tail at the same time (Huxley and Hogben 1922; Zavadovsky and Zavadovsky 1926; Haffner 1927).

Another principle was metabolic activation and increased energy expenditure. Kreitmair (1928) standardized thyroid preparations using the **weight loss of guinea pigs** after one week treatment as parameter. A guinea pig unit was defined as the dose which reduces the body weight of guinea pigs with an initial weight of 250–300 g within 7 days by at least 10%.

A different functional role is subserved by calcitonin. The **hypocalcemic hormone calcitonin**, was discovered by Copp (1962, 1964). Its effects are generally opposite to those of the parathyroid hormone. Calcitonin originates from parafollicular C-cells of the thyroid. The bioassay of calcitonin preparations is performed by assessing their ability to **lower the plasma calcium** in the rat. Assay of serum calcitonin has a significant diagnostic role for thyroid carcinoma.

Thyroid hormone receptors

Nuclear triiodothyronine binding proteins were purified and characterized by Torresanai and Anselmet (1978). Ichikawa and DeGroot (1987a,b) described purification and characterization of rat liver nuclear thyroid hormone receptors and thyroid hormone receptors in a human hepatoma cell line. Apriletti et al. (1988) reported large scale purification of the nuclear thyroid hormone receptor from rat liver and sequence-specific binding of the receptor to DNA. Ichikawa et al. (1988), Ichikawa and Hashizume (1991) published methods of an aqueous two-phase (dextran and poly-ethylene glycol) partitioning study of nuclear thyroid hormone receptors. Glucocorticoids, other steroid hormones, thyroid hormones and vitamin-derived hormones (including retinoids) all exert their effects by the regulation of hormone-responsive target genes within the cell nucleus. William and Franklyn (1994) reviewed the physiology of the steroid-thyroid hormone nuclear receptor superfamily. A nuclear hormone receptor-associated protein that inhibits transactivation by the thyroid hormone and retinoic acid receptors was described by Burris et al. (1995). Two different genes encode two different thyroid hormone receptors, thyroid hormone receptor-α and thyroid hormone receptor-β, and these two thyroid hormone receptors are often co-expressed

at different levels in different tissues. Chiellini et al. (1998) designed a high-affinity subtype-selective agonist ligand for the thyroid hormone receptor-β. The expression of thyroid hormone receptor isoforms in rat growth plate cartilage *in viv*o was described by Ballock et al. (1999). Yuan et al. (1998) described a component of a thyroid hormone receptor-associated protein (TRAP) coactivator complex which interacts directly with nuclear receptors in a ligand-dependent fashion. The sequence of the thyroid hormone response element and the recruitment of retinoid X receptors for thyroid hormone responsiveness was investigated by Wu et al. (2001).

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N.5.0.2 Thyroidectomy

PURPOSE AND RATIONALE

Endocrinological experiments for pharmacological evaluation of thyroid hormones and analogues may be performed in thyroidectomized rats. Bomskov (1937) described the method of thyroidectomy in various animal species, such as tadpoles, frogs, birds, goats, dogs, cats, rabbits, guinea pigs, rats and mice, based on the clinical experience with thyroid resection in humans.

PROCEDURE

The thyroid in rats consists of three lobes (left, median and right). The rat is anesthetized with Nembutal and the legs fixed on an surgical table. The fur of the neck is removed with electric clippers and the area disinfected. A median skin incision of 2.0 cm length is made. On both sides large salivary glands and maxillary lymph nodes are found. They are pushed aside, making visible the musculus hyoideus covering the trachea. This muscle is split in the midline. The isthmus of the thyroid connecting both lobes is located

below the thyroid cartilage. The lobes and the isthmus are separated with blunt forceps from the trachea and the blood vessels ligated. Alternatively, the thyroid can be removed by electrocauterization. In most cases, also the parathyroid glands are severed and postoperative substitution with calcium lactate 1% in drinking water is advised.

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N.5.1
In vivo tests for thyroid hormones

N.5.1.1
Oxygen consumption

PURPOSE AND RATIONALE

Basal metabolic rate, oxygen consumption and CO_2 production are increased by thyroid hormones. This has been used for diagnostic procedures in man as well as for evaluation of thyroid hormones and their derivatives in animals (indirect calorimetry). The very early method based on survival time of mice placed individually into tightly closed glass jars (Smith et al. 1947; Basil et al. 1950; Gemmill 1953) has been modified by methods which allow survival of the animals, measuring time until occurrence of convulsions. The assays are no longer applied.

PROCEDURE

Mice are placed individually into 200 ml wide-necked bottles. Groups of 10 mice are used for each dose of test preparation or standard. The bottom of the bottles is covered with filter paper to soak up the urine. The bottles are tilted to an angle of 60° and rotated 5 times per min in a special apparatus. The time until asphyctic seizures occur is noted. Immediately after observation of seizures, the mouse is released for recovery. Due to the defined muscle work, the time to seizures is shortened in controls to 20–30 min.

EVALUATION

Average time to seizures is calculated for each dosage and dose-response curves are established.

MODIFICATIONS OF THE METHOD

Several apparatuses have been designed to measure oxygen consumption in animals, e.g., by Holtkamp et al. (1955).

Stock (1975) described an automatic, **closed-circuit oxygen consumption apparatus** for small animals. A Perspex animal chamber is surrounded by a water jacket except for one end, which has a removable cover plate. This cover, as well as allowing access to the chamber interior, also holds the connections for the oxygen delivery line and the pressure line. Forexperiment involving injections, infusions and blood sampling, catheters are passed through, and sealed into rubber bungs which are then forced into holes in the cover plate. A rubber gasket forms an airtight seal between the cover and the chamber. Within the chamber, the animal is supported on a wire grid over a layer of self-indicating soda lime and silica gel. A major determinant of sensitivity in this system is the dead space of the chamber. Chambers with internal dimensions of $20 \times 10 \times 10$ cm are suitable for animals such as mice and rats up to about 250 g body weight. Fixed volumes of oxygen are introduced into the chamber by an automatic syringe dispenser (Fisons Scientific) which draws pure oxygen from a spirometer through a drying tube filled with silica gel. When chamber pressure exceeds atmospheric by about 3 mm H_2O, the microdifferential pressure switch (KDG Instruments) inactivates the dispenser. The dispenser is reactivated when the pressure differential drops below this threshold value. The volume of oxygen dispensed is adjusted to the smallest volume that, with a single action of the syringe, will return chamber pressure to above the threshold value. The particular dispenser used in this system has the advantages of being (a) gas tight and (b) when activated will complete its pump cycle even if the chamber pressure exceeds the threshold value in midcycle. In this way, a discrete fixed volume of oxygen is delivered each

time it is activated. To obtain the rate of oxygen consumption it is merely necessary to record the pump rate. This is done using the travel of the syringe piston to close a microswitch connected to an event recorder.

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N.5.1.2 Inhibition of iodine release

PURPOSE AND RATIONALE

The release of ^{131}I from the thyroid in rats is inhibited by treatment with thyroxine (Wolff 1951), and the degree of inhibition is related to the dose administered (Perry 1951). This phenomenon was used to compare thyroid hormone derivatives with the standard thyroxine. It is superseded by direct quantitation of thyroid hormones by analytical methods e.g. radioimmunoassay and HPLC chromatography, and by measuring feedback inhibition directly via the decrease in serum TSH.

PROCEDURE

Male Sprague-Dawley rats weighing 180–240 g are fed a commercial laboratory chow without or with addition of 0.03% propylthiouracil (reference compound for thyroid peroxidase inhibition). Food is withheld 8 h before and for 24 h after ^{131}I or ^{125}I is injected intraperitoneally at a dose of 25 μC. Under light ether anesthesia, radioactivity over the thyroid region of the neck is determined 40 h later. This value is taken as zero time and all further counts made at 24-h intervals are expressed as percent of zero time-counts after correction for physical decay of the isotope. After the reading at zero time, the diet is changed to a diet containing 0.03% propylthiouracil and various doses of the test preparation or the standard are injected subcutaneously at 24-h intervals for a total of four doses. The daily loss of ^{131}I is inversely proportional to the dose of thyroid hormone.

EVALUATION

Percent of zero time counts after 96 h of ^{131}I remaining in the thyroid after the last of 4 doses is plotted against logarithm of dose. From these dose-response curves, potency ratios are calculated.

This approach may be modified for short term uptake of ^{131}I or ^{125}I as a parameter of thyroid peroxidase inhibition by antithyroid drugs.

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N.5.1.3 Anti-goitrogenic activity

PURPOSE AND RATIONALE

Increased secretion of TSH induces thyroid enlargement and weight increase within a few days. In normal animals the secretion of TSH by the pituitary is regulated by feedback of thyroid hormones. The administration of goitrogenic compounds which block thyroid hormone synthesis and/or secretion reduces the concentrations of circulating thyroT4/T3 and their pituitary effect, thus releasing TSH from its feedback inhibition. The TSH rise

induces a hyperplasia of the thyroid follicles as indicated by an increase of thyroid weight. Hyperplasia is prevented by injection of thyroxine or thyroid hormone analogs.

PROCEDURE

Male Sprague-Dawley rats weighing 150–180 g are used in groups of 8–10 animals. During the period of treatment, 0.1% propylthiouracil (PTU) is added to the food or to the drinking water, or equivalent doses are given by gavage. Over a period of 2 weeks, the rats are treated (subcutaneously or by gavage) with various doses of the test compound or the thyroxine standard (10–40 µg/kg). PTU controls receive PTU and saline injections only. The rats are sacrificed after 14 days, their thyroid glands are dissected out cleanly and weighed rapidly to avoid evaporation loss. Thyroids may also be lyophilized first to weigh dry matter. The two to three-fold increase of thyroid weight by PTU is reversed dose-dependently to normal values by thyroid active substances.

EVALUATION

Dose-response curves are plotted for test compounds and the standard and activity ratios calculated.

MODIFICATIONS OF THE METHOD

The effect of PTU is monitored by measuring TSH, T4 and T3 and the inhibition of the TSH rise by thyroid substances is used as the parameter of goiter prevention.

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N.5.1.4 Tensile strength of connective tissue in rats, modified for thyroid hormones

PURPOSE AND RATIONALE

The decrease of tensile strength after a single injection of thyroid hormones is dose-dependent and can be used for evaluation of thyroid hormone derivatives. Short term treatment with corticosteroids increases the strength of connective tissue (Vogel 1969). This effect is antagonized by thyroid hormones (Ther et al. 1963; Vogel and Ther 1994). Thyroid hormones per se have a biphasic effect: short term treatment decreases the dose-dependent tensile strength of epiphyseal cartilage, tail tendons and skin strips, whereas treatment over 10 days increases these values, probably due to activation of endogenous adrenal secretion.

PROCEDURE

Male Sprague-Dawley rats weighing 110 ±10 g are injected subcutaneously with various doses of thyroid hormones (dose range of the standard L-triiodothyronine 0.1 to 1.0 mg/kg). After 24 h the animals are sacrificed and tensile strength of distal femoral epiphyseal plates, tail tendons, or skin strips are tested as described in Sect. N.2.1.3.2, N.2.1.3.3, N.2.1.3.4, and P.11.2.1.1.

EVALUATION

Doses-response curves of test compounds and standard are established and potency ratios calculated. T3 (L-triiodothyronine) is about 3 times more active than T4 (levothyroxine), in accordance with its metabolic activity.

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N.5.2 Antithyroid drugs

N.5.2.1 General considerations

Antithyroid drugs are characterized by their ability to interfere with synthesis, release, and/or peripheral action of the thyroid hormone, lowering the basal metabolic rate. Decrease of T4/T3 reduces thyroidal inhibition of the pituitary gland, TSH secretion increases and induces a goitrogenic response. This response was used for detecting antithyroid drugs and has been widely used for screening procedures. It is, however, non-specific and may be caused by several different mechanisms, including enzyme induction of glucuronyltransferases.

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N.5.2.2 Inhibition of iodine uptake into the thyroid of rats

PURPOSE AND RATIONALE

Propyl-thiouracil (PTU) and a wide spectrum of drugs may inhibit thyroid hormone synthesis. Some of these drugs can be used for treatment of thyrotoxicosis. As a consequence of thyroid peroxidase inhibition, the iodine uptake and content of the thyroid is decreased. This phenomenon is dose-dependent and may occur at lower doses than those increasing thyroid weight in rats (McGinty and Bywater (1945). The historical parameter of iodine content was replaced by measuring uptake and release of ^{131}I.

PROCEDURE

Groups of male Wistar rats age 26–28 days, weighing 40–45 g, are placed into metabolism cages. They are fed normal diet, and potassium iodide is added to the drinking water. The test compounds or the reference standard are added in various concentrations to the diet over a period of ten days. The amount of compound which each rat receives is calculated from the total food consumption over 10 days and expressed as milligram daily per kilogram of body weight. After 10 days of treatment, the rats are sacrificed and the thyroids dissected free from adjacent tissue and capsule. The thyroid is weighed and iodine content determined. In daily doses between 0.1 and 10.0 mg/kg, thiouracil decreases the iodine content of the thyroid dose-dependent. Definitely higher doses are necessary to increase thyroid weight.

EVALUATION

Dose-response curves of test compounds are compared with those of the reference standard for calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Walker and Levy (1989) used implantable pellets of propylthiouracil to induce thyroid dysfunction in rats. Uptake of labelled iodine is measured instead of iodine content. Release of labelled iodine may be stimulated by protirelin (TRH) injection.

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N.5.2.3 Antithyroidal effect on oxygen consumption in iodine treated mice

PURPOSE AND RATIONALE

This is a historical bioassay based on oxygen consumption increased in acutely potassium iodide treated mice resulting in a decrease of asphyxiation time (thyroid activation). The effect is antagonized by antithyroidal compounds. The methods for increased oxygen consumption after thyroid hormones (N.5.1.1) can be applied.

MODIFICATIONS OF THE METHOD

Rabbits treated with goitrogenic compounds or fed exclusively with cabbage (Chesney et al. 1928; Marine et al. 1929) show an up to tenfold increase of thyroid weight, histologically manifested as hyperplasia without colloid formation. These phenomena can be reversed by iodine treatment (Bomskov 1937).

Goiter formation as side effect of non-steroidal anti-inflammatory drugs was studied by Müller et al. (1985).

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N.5.3 Calcitonin

N.5.3.1 General considerations

The calcitropic hormone calcitonin, was discovered by Copp (1962, 1964, 1994). The hypocalcemic hypophosphatemic principle of the thyroid gland was termed thyrocalcitonin by Hirsch et al. (1964), Munson and Hirsch (1966), Raisz et al. (1967). Its effects are generally opposite to those of the parathyroid hormone. Calcitonin originates from parafollicular C-cells of the thyroid. Calcitonin secretion can be evaluated using the isolated perfused porcine thyroid (Pento 1985). Radioimmunoassays for calcitonin are available (Tashjian and Voelkel 1979), species specific methods for calcitonin determination need to be considered. Assays for calcitonin receptors have been described (Nissenson et al. 1985). A survey on the effects of exogenous calcitonin was given by Deftos (1989). The biology and clinical relevance of calcitonin gene peptides has been reviewed by Zaidi et al. (1990).

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N.5.3.2 Decrease of serum calcium in rats

PURPOSE AND RATIONALE

The bioassay of calcitonin preparations is performed by assessing their ability to lower the plasma calcium in the rat. This procedure has also been adopted by pharmacopoeias, using the International Reference Preparation for Calcitonin (porcine), consisting of freeze-dried purified pork calcitonin, or the International Reference Preparation for Calcitonin (salmon) consisting of freeze-dried purified synthetic salmon calcitonin. These assays, however, are replaced for calcitonin quantitation by physicochemical method for pharmaceutical quality control. Either intravenous or subcutaneous administration can be chosen. International standards for salmon calcitonin, eel calcitonin, and the Asu^{1-7} analogue of eel calcitonin have been elaborated (Zanelli et al. 1990). A second international standard for porcine and human calcitonins has been established by an international collaborative study group based on the *in vivo* rat hypocalcemia bioassay (Zanelli et al. 1993).

PROCEDURE

Groups of at least 5 female Wistar rats, weighing 100 to 120 g, are used. Three doses of standard preparation (usually 1, 3 and 9 milliU per rat) and 3 doses of test preparation are injected intravenously. Exactly 1 h after injection, blood is withdrawn under light anesthesia. Plasma calcium is determined by flame photometry or by atomic absorption photometry.

EVALUATION

Dose-response curves of decreases in plasma calcium are established and potency ratios with confidence limit are calculated.

MODIFICATIONS OF THE METHOD

Yates et al. (1990) assessed the acute hypocalcemic responses to single subcutaneous injections of calcitonin preparations in intact young male ICR Swiss mice weighing 12–20 g.

Calcitonin of the stingray and of the goldfish were characterized by Sasayama et al. (1992, 1993).

Kapurniotu and Taylor (1995) performed *in vitro* hypocalcemic assays in mice by analysis of serum calcium 1 h after sc. injection of lactam-bridged analogues of human calcitonin.

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N.5.3.3
Effect of calcitonin on osteoclasts *in vitro*

PURPOSE AND RATIONALE

Calcitonin acts primarily by inhibition of osteoclastic bone resorption (Friedman and Raisz 1965; Aliapoulios et al. 1966). Zaidi et al. (1990) reported the development and validation of three microbioassays for calcitonin based on calcitonin-induced inhibition of the activity of isolated osteoclasts.

PROCEDURE

Femora and tibiae are removed from newborn Wistar rats. The bones are freed from adherent soft tissues and cut across their epiphyses in HEPES-buffered Medium 199 supplemented with heat-inactivated fetal calf serum, benzyl penicillin (100 μU/ml) and streptomycin (100 μg/ml). The osteoclasts are mechanically disaggregated by curetting the bones of each rat with a scalpel blade into 1 ml medium and agitating the suspension with a pipette. Larger fragments are allowed to settle for 10 s, before the supernatant is dropped onto appropriate substrate (bone slices, plastic Petri dishes or glass coverslips).

Motility-based system

The morphological appearance of stained osteoclasts is used as an index to assess the state of cytoplasmic activity. Osteoclasts are settled on coverslips in microtiter wells and are incubated for 20 min at 37 °C. The coverslips are removed, washed with Medium 199 and placed in separate wells, each containing 100 μl medium.

Following a further incubation for 30 min (37 °C), serial dilutions (10-fold) of salmon or human calcitonin or test preparations or appropriate dilutions of plasma samples are added. The cells are finally incubated for 2 h, fixed in 10% glutaraldehyde and stained with toluidine blue. The state of motility of each osteoclast on each coverslip is scored by observing the characteristic shape change these cells undergo when motility is inhibited; a motile cell is characterized by a smooth outline with increased staining intensity over all or part of its periphery, whereas a immotile cell typically shows an irregular pale outline without ruffled edges. The number of immotile cells is counted on each coverslip and expressed as a percentage of the total number of cells counted.

Cytoplasmatic spreading system

Osteoclasts are settled in tissue culture dishes (35 mm) and are incubated at 37 °C for 20 min to allow sedimentation and attachment. The cells are then washed with Medium 199 and 2 ml of the same medium is placed in each well. The dishes are placed in the incubation chamber of an inverted phase-contrast microscope. Images of the osteoclasts are recorded on a time-lapse video recorder. A tracing of their outlines is transferred through a digitizing system into a computer, programmed to measure area within each tracing.

The outlines of each osteoclast are recorded before or after the addition of calcitonin or vehicle to the cultures. For each variable, the outline of 6 osteoclasts is traced after 60 min incubation in the chamber and again 40 min following the addition of the hormone. The mean surface area covered by 6 osteoclasts after incubation is expressed as a percentage of the mean surface area of the osteoclasts before the addition of hormone or vehicle.

Bone resorption system

Specimens of human femoral cortical bone are obtained from patients who died without evidence of bone disease. The adherent soft tissue is removed and the bone cortex cut longitudinally into slices (0.1 mm thick). The slices are then cut into pieces (approximately 3 mm^2). They are cleaned by ultrasonication (15 min, in sterile distilled water), dehydrated by immersion in 80% aqueous ethanol for 2 h and stored to dry at room temperature. Osteoclasts isolated in Medium 199 are dropped onto 12–16 bone slices placed in a well of an 18-mm multiwell dish. Following incubation (37 °C, 15 min), slices are removed, washed gently in Minimal Essential Medium supplemented with 10% FCS and antibiotics as described above. They are placed in separate wells, each well containing 5–6 slices in 900 µl medium. After further incubation (37 °C, 10% humidified CO_2, 10 min), 100 µl of medium containing the test concentration of the hormone or the test solution is added. Human $PTH_{(1-34)}$ (0.1 U/ml) is used to assess functional effects of contaminating osteoblasts.

The calcitonin analogs are tested at various concentrations (10-fold dilutions). Finally, bone slices are incubated overnight (37 °C, 10% humidified CO_2, 18 h). The cells are fixed in glutaraldehyde, stained with toluidine blue and examined by transmitted light microscopy. Osteoclasts and mononuclear cells are counted. The slices are then bleached by immersion in sodium hypochlorite solution for 30 min and dehydrated in 80% aqueous ethanol. Finally, they are sputter coated with gold, randomized and examined in a scanning electronic microscope. The numbers of osteoclastic excavations, each defined by a continuous border, are counted. The area of bone surface resorbed is calculated by tracing the outline of the concavities into a digitizing tablet, linked to a microcomputer. Resorption surface areas may be expressed as a percentage of the mean of the control response.

EVALUATION

Data of each assay are analyzed using classical methods for analysis of parallel line assays. Estimates of relative potencies are calculated from parallel log dose-response lines of test preparations and reference preparation.

Osteoclasts were mechanically disaggregated from neonatal rat long bones and dispersed at low densities on slices of devitalized bovine cartilage bone. The resulting areas of bone excavation were quantified with micrometric precision by scanning electron microscopy together with computer-assisted image analysis. These findings were used to develop a formal bioassay for calcitonin.

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N.5.3.4 Receptor binding and cAMP accumulation in isolated cells

PURPOSE AND RATIONALE

The human breast cancer cell line T47D responds to calcitonin and its analogs by receptor binding and accumulation of cAMP. This can be used as biological assay (Findlay et al. 1980, 1983, 1985; Grauer et al. 1992; Sexton and Hilton 1992; Blind et al. 1993).

PROCEDURE

The human breast cancer cell line T47D was originally established from a pleural effusion from an infiltrating ductal breast cancer (Horwitz et al. 1978). For binding experiments, cell monolayers are washed with 0.02% EDTA before treatment with 0.125% trypsin in 0.02% EDTA for 2 min at 37 °C, addition of complete medium before centrifugation at 200 *g*, and resuspension in complete medium.

Iodination of calcitonin is performed with ^{125}I using the chloramine-T method.

For binding experiments T47D cells suspended in isotonic buffer are added to ^{125}I-labeled salmon calcitonin mixed with varying concentrations of unlabeled calcitonin or analogs and incubated at 20 °C for 1 h. Nonspecific binding is assessed as the binding of ^{125}I-labeled salmon calcitonin in the presence of excess (2 µg/ml) unlabeled salmon calcitonin.

Stimulation of adenylate cyclase in intact T47D cells by calcitonin analogs is assessed by measuring

[^{3}H]cAMP production in cells prelabeled with [^{3}H]adenine. Cellular ATP-pools are labeled by incubation with 2,8-[^{3}H]adenine (0.5–2 µCi/ml) for 2 h at 37 °C in 12-well culture dishes in RPMI 1 640 medium containing 0.1% BSA. Cells are then washed twice with serum-free medium and incubated for a further 20 min in medium containing 0.1% BSA and 1 mM isobutylmethylxanthine IBMX before treatment with calcitonin and its analogs for 10 min at 37 °C in the same buffer. Incubations are terminated by removing medium and adding 100 µl 20% trichloroacetic acid at 4 °C. This is followed by 800 µl 5 mM solution of ATP, ADP, AMP, cAMP, and adenine. The [^{3}H]cAMP is isolated by chromatography on Dowex and alumina. Radioactivity is counted in a scintillation counter.

EVALUATION

For both parameters full dose-response curves are generated and the concentrations required for half-maximal responses are calculated.

MODIFICATIONS OF THE METHOD

Yates et al. (1990) measured stimulation of adenylate cyclase activity by calcitonin analogs in primary cultures of mouse renal cortex which were prepared according to the methods of Fukase et al. (1982) from 4-week-old ICR Swiss mice and used at confluence after 4 days of culture.

A radioreceptor assay for potency determinations of formulations of salmon calcitonin was described by Sjödin et al. (1990).

Albrandt et al. (1993) cloned two receptors with high affinity for salmon calcitonin from the nucleus accumbens region of rat brain.

Likewise, Sexton et al. (1993) identified in rats two isoforms of the calcitonin receptor, designated C1a and C1b.

Functional aspects of the isoforms C1a and C1b were discussed by Martin et al. (1995).

The calcitonin receptor isoforms C1a and C1b were localized in rat brain using *in vitro* autoradiography by Hilton et al. (1995).

Keustner et al. (1994) cloned and characterized a second form of the human calcitonin receptor from T47D cells.

Sexton et al. (1994) assayed the cloned renal porcine calcitonin receptor cDNA expressed by transient transfection in COS-1 cells or stable transfection in HEK-293 cells for interaction with calcitonin, amylin and calcitonin gene-related peptide. The results suggested that amylin may act as a natural ligand for the renal porcine calcitonin receptor.

The various pathways in signal transduction by calcitonin were discussed by Horne et al. (1994).

Houssami et al. (1994) found that different structural requirements exist for calcitonin receptor binding specificity and adenylate cyclase activation.

Suva et al. (1997) synthesized benzophenone-containing calcitonin analogs and tested them for receptor binding and stimulation of cAMP accumulation.

Povzek et al. (1997) investigated the structure/function relationship of salmon calcitonin analogues as agonists, antagonists, or inverse agonists in heterologous calcitonin receptor expression systems using 2 calcitonin receptor cell clones, B8-H10 and G12-E12, which express about 5 million and 25 000 C1b receptors/cell, respectively.

The location of the phosphorylation of the human calcitonin receptor by multiple kinases was studied by Nygaard et al. (1997).

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N.6 Parathyroid hormone

N.6.0.1 General considerations

The primary function of the parathyroid hormone (PTH) is to maintain a constant concentration of Ca^{2+} in the extracellular fluid. Processes that are regulated include the absorption of Ca^{2+} from the gastrointestinal tract, the deposition and mobilization of bone Ca^{2+}, and the excretion of Ca^{2+} in urine, feces, sweat, and milk. PTH is the functional antagonist of calcitonin. The most prominent effect is to promote the mobilization of Ca^{2+} from bone. In the kidney, tubular re-absorption of Ca^{2+} is increased and tubular re-absorption of phosphate is inhibited. Clinically, idiopathic or postoperative hypoparathyroidism results in hypocalcemia followed by tetany.

PTH can be used for treatment of hypoparathyroidism, but the symptoms of the disease can also be effectively antagonized by administration of dihydrotachysterol. A survey on the structure and function of the parathyroid gland in animals has been published by Capen and Rosol (1989). The biologically active synthetic human parathyroid hormone 1–34 fragment (active sequence of PTH) is used for diagnostic testing (Mallette 1988).

An experimental model for secondary hyperparathyroidism with elevated levels of parathyroid hormone in rats has been described by Sancho et al. (1989).

Assays for parathyroid hormone receptors have been described (Habener and Potts 1976; Nissenson et al. 1985; Schneider et al. 1993).

Immunoassays for PTH are those measuring intact hormone (N-terminal, intact) and those measuring inactive fragments and partial sequences of the intact hormone (mid-region, C-terminal, polyvalent) (Enders et al. 1989). An immunochemiluminometric assay has been described by Klee et al. (1992).

Parathyroid hormone-related protein (PTHrP) has been first identified and cloned from malignant tumor cells and tissues from patients with the syndrome of humoral hypercalcemia of malignancy (Moseley et al. 1987; Strewler et al. 1987; Suva et al. 1987). PTHrP-(1-34) and PTH-(1-34), act via a single species of cloned receptor (Abou-Samra et al. 1992; Schipani et al. 1993) although other studies have shown that specific receptors for each of these peptides exist (Usdin et al. 1995; Behar et al. 1996; Bergwitz et al. 1997; Yamamoto et al. 1997; Fukayama et al. 1998). PTHrP is an example for the widening array of sequences identified by molecular endocrinology, functional characterization of their endocrine role often following with a considerable delay.

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N.6.0.2
Receptor binding assay for PTH

PURPOSE AND RATIONALE

In many instances, the presence of receptors is now explored as the counterpart of the presence of hormonally active substances in tissues and organs. Three receptors that are activated by PTH have been cloned. PTH and PTHrP bind to the PTH_1 receptor, for which many studies are published, e.g., (Abou-Samra et al. 1992; Uneno et al. 1992; Schipani et al. 1993; Ureña et al. 1993; Kaufmann et al. 1994; Schermer et al. 1994; Orloff et al. 1995; Usdin et al. 1995; Bergwitz et al. 1996; Gardella et al. 1996; Yasuka et al. 1996; Bergwitz et al. 1997; Bisello et al. 1997; Guo et al. 1997; Yamamoto et al. 1997; Fukayama et al. 1998; Yaghoobian and Drueke 1998).

Behar et al. (1996b) reported ligand binding in stably transfected human embryonic kidney cells, HEK-293/C-21 cells that express the hPTH/PTRrP receptor and HEK/BP16 cells that express the hPTH2 receptor.

The PTH_2 receptor has about 50% amino acid sequence identity with the PTH_1 receptor and is not activated by PTHrP, but by a peptide isolated from bovine hypothalamus, TIP39, which is only distantly related to PTH and PTHrP (Usdin 2000).

The PTH_3 receptor has been isolated from zebrafish (Rubin and Juppner 1999).

PROCEDURE

Stably transfected human embryonic kidney cells, HEK-293/C-21 cells that express the hPTH/PTRrP receptor (Pines et al. 1994) and HEK/BP16 cells that express the hPTH2 receptor (Behar et al. 1996a) are maintained in DMEM supplemented with 10%FBS.

The cells are incubated with [^{131}I]PTH-(1-34) (100 000 cpm/well) with or without competing unlabeled PTH-(1-34) or other ligands in binding buffer for 2 h at room temperature. Cells are washed twice with PBS, then solubilized in 0.5 ml 0.1 M NaOH. Aliquots are taken for determination of bound radioactivity by γ-counting.

EVALUATION

Specific binding is expressed as counts per min bound/well and as the percent specific binding of radioligand. Affinity constants and binding capacity of these receptors in tissue may be calculated, and dose-response curves of ligands may be compared.

MODIFICATIONS OF THE METHOD

McCuiag et al. (1994) reported the molecular cloning of the gene encoding the receptor for the parathyroid hormone/parathyroid hormone-related peptide in the mouse.

Inomata et al. (1995) characterized a parathyroid hormone (PTH) receptor with specificity for the carboxy-terminal region of PTH-(1-84).

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N.6.0.3
PTH assay by serum calcium increase

PURPOSE AND RATIONALE

The classical method by Collip and Clark (1925) involved the measurement of the rise in serum calcium after administration of parathyroid extracts to dogs. Hamilton and Schwartz (1932) used rabbits treated

with oral loads of calcium chloride. The intact rat is very insensitive to injected parathyroid hormone; however, parathyroidectomy produces an increase in sensitivity (Davies and Gordon 1953; Davies et al. 1954).

PROCEDURE

Male Wistar rats weighing 200–250 g are anesthetized with pentobarbital sodium i.p. Parathyroidectomy is performed by cauterization. After a recovery period of one week, blood is withdrawn by retroorbital puncture (baseline). Various doses of the test preparation or standard are injected subcutaneously to groups of 6–10 animals. Blood samples are obtained again 21 h later. Serum calcium is determined by flame photometry. The increase of calcium 21 h after PTH injection is calculated for each animal.

EVALUATION

Mean values of the increase in serum calcium are plotted versus logarithm of dose for the test preparation and for the standard calculation of dose-response and potency ratios.

MODIFICATIONS OF THE METHOD

An increase of whole body calcium and skeletal mass after treatment with parathyroid hormone in normal rats and in rats with osteoporosis induced by pregnancy and lactation under a low calcium diet was found by Hefti et al. (1981).

Parathyroid hormone prevented bone loss and augmented bone formation in ovarectomized rats (Kalu et al. 1990; Liu and Kalu 1990).

The active sequence of PTH has been used instead of the full secreted sequence.

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N.6.0.4 Serum phosphate decrease after PTH

PURPOSE AND RATIONALE

Tepperman et al. (1947) developed a method using the fall in serum inorganic phosphorus in the rat after injection of parathyroid hormone.

PROCEDURE

Male Wistar rats weighing 150–200 g are fed Purina dog chow for at least 2 weeks prior to the experiment. During the experiment only water is allowed. Blood samples (baseline and PTH stimulated) are taken from the tail and 0.6 ml is collected from each rat into tubes, centrifuged for 10 min and 0.2 ml samples of the serum are pipetted into 6 ml of 10% trichloroacetic acid; this is centrifuged and 5 ml aliquots of the protein-free solution are used for the estimation of inorganic phosphorus, e.g., by the method of Fiske and Subbarow (1925). Serum phosphorus is measured before and 3 h after subcutaneous administration of various doses of test preparation or standard.

EVALUATION

Dose-response curves showing a linear relationship of log dose and response are suitable for the calculation of potency ratios.

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N.6.0.5 cAMP release in isolated perfused rat femur

PURPOSE AND RATIONALE

This is an *in vitro* assay. The effect of parathyroid hormone and analogs on release of cAMP from adult bone can be measured in a perfusion system of isolated rat femora (Sugimoto et al. 1985; Lopez-Hilker et al. 1992).

PROCEDURE

Five-week-old Wistar rats anesthetized with pentobarbital (45 mg/kg) are heparinized, and the femora are removed. Adhering muscles are stripped from the bone. A hole with about half of the depth of the cortex is made with a fine drill at the nutrient foramen below the femoral neck. Then a 21-gauge needle is inserted into this hole and fixed by dental cement to avoid leakage of the perfusate. The bone is then placed in an apparatus for liver perfusion and perfused at a flow rate of 1 ml/5 min by a pump with Krebs-Ringer-bicarbonate, continuously gassed with 95% O_2 and 5% CO_2 and containing 1 mg/ml glucose. Once the perfused bone is assembled, the bone is allowed to equilibrate for 45 min. Samples are collected into a chilled tube for the last 5 min for determination of basal cAMP levels. Then various doses of the test preparations or the standard are infused for 5 min and serial samples are collected every 5 min. In the perfusate, cAMP is measured by radioimmunoassay.

EVALUATION

Time-response curves and dose-response curves are established allowing the calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Nissenson et al. (1981) measured the activation of canine renal cortical plasma membrane adenylate cyclase activity, produced by parathyroid hormone standard and test sera from parathyroid venous effluent of patients with primary hyperparathyroidism, in the presence of the hydrolysis-resistant GTP analog, 5'-guanylimidodiphosphate.

Gundberg et al. (1995) compared the effects of parathyroid hormone and parathyroid hormone-related protein on osteocalcin release in the isolated rat hindlimb and in intact and thyroparathyreoidectomized rats.

Saito et al. (1987) established a new biological assay system for simultaneous measurement of bone resorption and bone mineralization in **organ cultures of chick embryonic femur**. Eleven-day-old chick embryonic femur was labeled with ^{45}Ca *in vitro*. $T_{1/2}$ of calcium efflux was calculated from the sequential release of the label into the medium. Parathyroid hormone increased calcium mobilization indicating enhanced bone resorption, whereas hydrocortisone and sodium fluoride inhibited bone resorption. Calcitonin was ineffective.

Barling et al. (1989) measured the adenylate cyclase response to parathyroid hormone in cultured rabbit marrow fibroblast cells.

Docherty and Heath (1989) used osteosarcoma cells for an *in vitro* bioassay determining cAMP formation.

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N.6.0.6
Renal and metatarsal cytochemical bioassay

PURPOSE AND RATIONALE

Cytochemical bioassays using renal and metatarsal tissue are sensitive enough to detect plasma levels of parathyroid hormone and useful to determine the agonist and antagonist activities of fragments and analogues (Chambers et al. 1978; Goltzman et al. 1980; Bradbeer et al. 1988; Loveridge et al. 1991; Zaman et al. 1991).

PROCEDURE

For the **renal cytochemical assay**, kidney segments from vitamin D-depleted guinea pigs are maintained in non-proliferative organ culture for 5 h using Trowell's T8 medium (GIBCO). The medium is then changed for 8 min before exposure to various doses of the parathyroid hormone standard, the parathyroid hormone fragment or the analog for an additional 8 min. In the experiments for antagonistic activities, each segment is exposed to a single concentration of hPTH-(1-84) (106 fmol/liter) or to hPTH-(1-34) (255 fmol/liter) in the presence or absence of the antagonist to be tested. The segments are then shock-frozen to –70 °C in N-hexane before being sectioned at 16 µm on a cryostat. The sections are examined for glucose 6-phosphate dehydrogenase activity using a

specific staining. The precipitated formazane is quantified in the cells of the distal convoluted tubules by means of a microdensitometer (wavelength 585 nm). Ten readings with each of two duplicate sections are made and the results presented as the mean integrated absorbance × 100 ±SEM.

For the **metatarsal cytochemical assay**, the metatarsals of young female Wistar rats weighing 50–100 g are removed and the growth plates are isolated. Only the four longest metatarsals have growth plates large enough to be of use. The metatarsals are maintained individually in nonproliferative organ culture in 5–10 ml Trowell's T8 medium buffered to pH 7.6 in an atmosphere of 95% O_2, 5% CO_2 at 37 °C for 5 h. After the culture period, the medium is removed and each metatarsal exposed to fresh medium (buffered to pH 7.6 by bubbling with a 95% O_2, 5% CO_2 mixture) containing a low priming dose of PTH (0.5 fg/ml) for 8 min, followed by exposure to known concentrations of a standard PTH preparation or various concentrations of the analog or to dilutions of plasma for 8 min. The metatarsals are then briefly dipped in a 5% solution of polyvinyl alcohol and chilled immediately in N-hexane to –70 °C.

Each bone is sectioned at 10 µm in a cryostat. The sections are reacted for glucose 6-phosphate dehydrogenase activity in a similar way as described for the renal cytochemical assay. The activity in hypertrophic chondrocytes is linear with respect to time from 10 min, at which time there is enough formazan to be measured, up to 40 min when the density of the formazan formed is too great for a reliable linear response. The enzyme activity in each section is measured in 10 individual hypertrophic chondrocytes or osteoblasts lining the metaphyseal trabeculae by scanning and integrating microdensometry at a wave length of 585 nm. The results are presented as the mean integrated extinction × 100 ±SE of 10 measurements from each of two sections of each metatarsal.

EVALUATION

Dose-response curves are tested for linearity and parallelism and potency ratios are calculated.

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N.6.0.7 cAMP accumulation in cultured cells

PURPOSE AND RATIONALE

Parathyroid hormone and parathyroid hormone-related protein (PTHrP) stimulate dose-dependent intracellular cAMP accumulation in various cell types, such as in PTHrP-overexpressing ROS cells, a osteoblast-like cell line Motumura et al. (1996); in UMR106 cells, a rat osteosarcoma cell line (Oldenburg et al. 1996); in SaOS cells, a human osteosarcoma cell line (Rodan et al. 1987; Fukayama and Tashjian 1994); in COS-7 cells transfected with the human PTH-1 (PTH/PTHrP) receptor and the human PTH-2 receptor (Bergwitz et al. 1997); or in stably transfected human embryonic kidney cells (HEK-293/C-21 cells that express the hPTH/PTRrP receptor and HEK/BP16 cells that express the hPTH2 receptor) (Behar et al. 1996b).

PROCEDURE

Stably transfected human embryonic kidney cells, HEK-293/C-21 cells that express the hPTH/PTRrP receptor (Pines et al. 1994) and HEK/BP16 cells that express the hPTH2 receptor (Behar et al. 1996a) are maintained in DMEM supplemented with 10%FBS. For cAMP determination, C-21 or BP-16 cells are incubated in 24-well tissue plates with various peptides for 10 min in DMEM in the presence of 1 mM 3-isobutyl-1-methylxanthine. The incubation is terminated by removal of the cell culture medium and addition of perchloric acid (final concentration 30%, vol/vol). Samples are neutralized with potassium bicarbonate and acetylated with acetic anhydrate. Total cAMP values (medium plus cells) are determined by RIA. Data are presented as the percent stimulation, from the ratio of maximal accumulated cAMP levels obtained in the presence of the highest concentration of the agonist PTH-(1-34) compared to the basal cAMP level. The accumulated cAMP levels

(picomoles) are calculated per 100 000 cells. Cell number is determined in a Coulter counter.

EVALUATION

Data are presented as the triplicate mean of ($[cAMP]_c$/$[cAMP]_{PTHmax}$ ±SEM) × 100 or sample stimulation vs. maximal PTH induced stimulation. $[cAMP]_c$ is the concentration of cAMP accumulated in response to a given concentration of a ligand, and $[cAMP]_{PTHmax}$ is the concentration of cAMP accumulated at the maximal dose (10^{-6} M) of PTH-(1-34).

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N.6.0.8 Anabolic activity in ovariectomized, osteopenic rats

PURPOSE AND RATIONALE

This is an *in vivo* bioassay. Vickery et al. (1996) tested an analogue of human parathyroid hormone-related protein (1–43) in ovariectomized, osteopenic rats.

PROCEDURE

Groups of 3-month old virgin female rats are subjected to bilateral ovariectomy or sham surgery. The ovariectomized animals are treated by daily subcutaneous injections of 0, 9, 29 or 80 µg/kg of test compound or human parathyroid hormone-related protein (1–43), starting on day 17 after surgery and continuing for 21 days. On day 12 and 19 following the first day of treatment, animals are dosed intraperitoneally with 6 mg/kg of a 3.75 mg/ml solution of calcein (Sigma Chemical Co.) in 2% sodium bicarbonate-saline. Body weights are recorded, following an 18-h fast, on the last day of treatment. The animals are sacrificed immediately following the last injection and the femurs, tibiae, and L2 vertebrae are excised.

The distal part of the right femur is cut in half longitudinally after removal of the epiphysis. The bone marrow is flushed out using a stream of water. The trabecular and cortical bone are separated using a dental drill. Calcium is extracted by immersion of the trabecular bone for 3 days and the cortical bone for 5 days in 5% trichloroacetic acid. The calcium content of the extracts is determined. Measurement are expressed as mean ±SEM in units of milligrams of Ca^{2+}/distal half femur/100 g of body weight.

For histomorphometry, the left tibiae from sham, ovariectomized and high dose-treated rats are removed and fixed in formalin. The tibiae are cut with a diamond disc into three pieces: the proximal 1 cm, 2 cm of the diaphysis, and the distal end. The proximal and diaphyseal portions are transferred to Villanueva stain for 72 h (Villanueva and Lundin 1989). The proximal specimen is then dehydrated through increasing concentrations of ethanol (70–100%), defatted in acetone, and embedded in modified methyl methacrylate. Pairs of 5-µm thick frontal sections are prepared from the anterior aspect with a microtome. The first section is stained with a modified Masson trichrome technique (Goldner 1938) and the second left unstained.

The diaphyseal portion is also dehydrated, defatted and embedded in methyl methacrylate. Five 150-µm-thick sections are prepared, ground to 100 µm thickness and mounted unstained.

Growth cartilage thickness is measured by finding the distance between the proximal border of the epiphyseal growth cartilage and metaphyseal junction at 12 places spaced 0.2 mm apart.

On each slide, a rectangular window is defined as the field for evaluation. The top line is drawn under the primary spongiosa, and the second line is drawn parallel to it and 3.5 mm distally. The side lines are drawn near to, but not touching, the endocortical surface.

Histomorphometric variables are calculated for mean trabecular volume and structure (trabecular thickness, number, spacing, mineralizing surface, individual osteoblast activity, and osteoclast surface). Surface-based, bone-volume based, and total tissue volume-based bone formation rates are also calculated.

In the cortical bone of the tibial diaphysis, the periostal and endocortical surfaces are measured separately. Using transmitted light at 25× magnification, the periosteal and endoosteal perimeters are measured. The periosteal single-labeled and double-labeled surface and endoosteal single-labeled and double-labeled surface are measured using 160× magnification.

Vertebral processing and electron microscopy is performed in sham, ovariectomized and compound treated groups. The second lumbar vertebra is removed, dissected from soft tissue, and split sagitally with a diamond saw. The 2 halves are fixed in a mixture of glutaraldehyde and formaldehyde for 24–48 h at 4 °C. The tissue is decalcified in 10% EDTA in 0.1 M cacodylate, pH 7.2, postfixed for 1.5 h in OsO_4, stained in block with uranyl acetate, dehydrated and fixed in Epon. Fifty nm sections are cut and examined in an electron microscope. Microphotographs are printed at a final magnification of 1 300×. Each microphotograph is overlaid with a grid with a total test line length of 2.22×10^3 µm. Each intercept of the line with the trabecular surface is scored into one of four categories, depending on the cell type adjacent to the intercept: osteoblasts, osteoclasts, lining cells, no cells. The proportion of the trabecular bone surface area covered by each cell type is estimated by pooling the pictures from each rat and summing the grid points that intersect each cell type, then dividing by the total number of intersection, excluding the "no cell" counts.

EVALUATION

The treatment groups are compared by one-way analysis of variance (ANOVA) followed by Fisher's least significance difference to compare each treatment group to ovariectomized vehicle control group. For the histomorphometric determinations, the Kruskal-Wallis test is applied for group differences. For relative surface area cell coverage estimation, the treatments are compared overall as to the proportions of osteoblasts, osteoclasts, lining cells; and no cells using one way ANOVA.

MODIFICATIONS OF THE METHOD

Anderson et al. (1990) proposed the ovariectomized, lactating Sprague-Dawley rat as an experimental model for the rapid development of osteopenia which may be used to test the effectiveness of bone-retentive drugs, potentially useful in treating osteoporotic women. Rats were ovariectomized on day 2 postpartum and were kept on a low calcium diet. Measurements of serum total calcium, ionic calcium, albumin and parathyroid hormone were conducted between days 4 and 21 of lactation.

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N.7
Anterior pituitary hormones

N.7.0.1
Hypophysectomy in rats

PURPOSE AND RATIONALE

This is a classical technique for the description of biological effects of pituitary hormones by substitution. Its usefulness is however limited by the fact that all pituitary hormones are eliminated at once. Various techniques of hypophysectomy have been described by Biedl (1916), Thompson (1932), Collip et al. (1933), Anselmino and Pecharz, (1935), Bomskov (1939) in several animal species, including dog, cat, rabbit, ferret, guinea pig, rat, mouse, chicken, and frog. At present, the technique of hypophysectomy in the rat remains of limited interest for biological standardization of anterior pituitary hormones. The parapharyngeal approach is less destructive than the transauricular approach, and is preferred for bioassay.

PROCEDURE

Male Wistar or Sprague-Dawley rats weighing 110–150 g are anesthetized with ether or modern anesthetics. Their legs are fixed to a surgical table. The head is stretched by a thread around the upper incisor teeth. The fur on the neck is removed with electric clippers. The field of surgery is cleaned with alcohol. A median skin incision is made 2.5 cm forward from the sternum. The large salivary glands and the maxillary lymph nodes

are pushed to the side, the muscles over the trachea are divided at the midline, and slightly below the thyroid a hole is made in the trachea with a needle ("tracheotomy"). Tracheal mucus is removed by vacuum aspiration. The insertion of a tracheal cannula is not necessary.

A deep blunt dissection is made directly medial to the tendon of the left or right digastric muscle. The ipsilateral glossopharyngeal nerve and the blood vessels are drawn to the side with a small blunt hook. The pharynx and the trachea are drawn to the other side by a second hook allowing the approach to the midline of the base of the skull.

The base of the skull is cleaned using small pellets of cotton-wool. The spheno-occipital suture running between both mastoid processes is prepared. From behind, the crista occipitalis runs longitudinally up to the middle of this suture. Just at this point, a burr hole is made through the basis of the scull with a dental drill. Care has to be taken to leave the surrounding sinus blood vessels intact. The hole is dried from blood with a cotton pellet. The pituitary can be seen as a small, lens-shaped organ. With a fine glass tube, attached to a vacuum pump over a Woulff bottle, the pituitary is carefully sucked out severing the hypophyseal stalk. Occasional bleeding is stopped with a cotton-wool pellet. The trachea is freed from mucus with the suction pump and the wound closed.

MODIFICATIONS OF THE METHOD

Instead of the parapharyngeal approach, the **transauricular approach** can be used (Jung and Vogel 1963). Male Wistar or Sprague-Dawley rats weighing 120–150 g are anesthetized with ether. The animal is placed with the right side on a glass plate. The head is fixed between the thumb and the index finger of the left hand. In perpendicular direction, a dental drill with a diameter of 2.2 mm is introduced into the meatus acusticus externus osseus. First, the fine lamellae of the middle ear are passed. Then, the drill is directed from the external ear to the contralateral orbita. On this way, the thin inner wall of the bulla tympani is perforated. At this place, the junction of bulla tympanica, os occipitale and os sphenoidale is located. A small, straight forceps is introduced to free the pituitary. Then, a needle with a diameter of 1.6 mm and with a blunted tip being attached to a glass cannula is introduced and the pituitary is removed by suction.

The procedure is less time-consuming than the parapharyngeal approach, but needs much more technical skill.

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N.7.1 Gonadotropins

N.7.1.0.1 General considerations

Follicle-stimulating hormone (FSH) and luteinizing-hormone (LH) are glycoproteins which exists in multiple molecular isoforms (microheterogeneity) with different molecular weights and with different degrees of glycosylation (Ulloa-Aguirre et al. 1988; Dahl and Stone 1991). This is however not reflected by the bioassay The immunological methods provide only limited information on biological activity and may measure intact hormone or inactive subunits depending on the assay. *In vivo* animal bioassays, e.g., the classical Steelman-Pohley assay, *in vitro* cell bioassays with a high degree of sensitivity and specific radioreceptors assays have been developed (Simoni and Nieschlag 1991). Striking differences between *in vivo* bioassays, *in vitro* bioassays, receptor bioassays, and immunoassays were found when the International

Standard for Pituitary FSH was assayed in terms of the Second International Reference Preparation of Human Pituitary FSH and LH (Storring and Gaines Das (1989).

Receptors for gonadotropins in various species have been cloned (Poyner and Hanley 1992).

Time-resolved fluoroimmunoassay for gonadotropins has been proposed by Iwasawa et al. (1994).

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N.7.1.1 Follicle-stimulating hormone (FSH)

Some of the methods described here are used for quantal assay of biological FSH activity (in animals and *in vitro*), others are used for the assay of FSH containing samples from experimental and clinical studies.

N.7.1.1.1 Ovarian weight in HCG-primed rats

PURPOSE AND RATIONALE

Follicle-stimulating hormone (FSH) increases the weight of ovaries in immature rats by inducing follicular maturation. This effect is greatly enhanced by simultaneous administration of a constant dose of human chorionic gonadotropin (HCG) for additional luteinization allowing the detection of low amounts of FSH (Steelman and Pohley 1953).

PROCEDURE

Immature female Sprague-Dawley rats weighing 40–45 g receive twice daily for 3 days subcutaneous injections of 3 different doses of the standard (e.g., ovine NIH-FSH-S-9, National Institute of Health, Bethesda, USA) or the test preparation both of them together with a total of 25 IU HCG (Primogonyl®, Schering AG, Berlin) dissolved in 2% gelatin solution in saline. Six to 8 animals are used per group. Eighteen hours after the last injection, the animals are sacrificed, the ovaries extirpated, freed from adherent fat and connective tissue, and weighed to the nearest 0.1 mg.

EVALUATION

Dose-response curves for standard and test compound are plotted and potency ratios with confidence limits calculated.

CRITICAL ASSESSMENT OF THE METHOD

The Steelman-Pohley test is rather specific for FSH. The addition of LH, TSH, ACTH, HGH, or prolactin did not influence the dose-response curves of FSH (Christiansen 1972b). The method has been adopted by pharmacopoeias, e.g., British Pharmacopoeia 1988.

MODIFICATIONS OF THE METHOD

Igarashi and McCann (1964) reported a bioassay for follicle stimulating hormone in mice using the synergism with HCG and uterine weight as endpoint.

Brown and Wells (1966) described an assay of human urinary follicle-stimulating hormone using HCG induced augmentation of ovarian weight in mice. The method has been used by Wide and Hobson (1986) studying the influence of the assay method used on the selection of the most active forms of FSH from the human pituitary.

Lamond and Bindon (1966) recommended the use of immature hypophysectomized mice on the basis of augmentation of FSH by HCG and uterus weight as endpoint as an assay for FSH.

Gans and van Rees (1966) studied a testicular augmentation assay method for follicle stimulating hormone. Immature male rats were hypophysectomized and on the same day the right testis was removed and weighed. Treatment with various doses of FSH together with a constant dose of 20 IU HCG was started on the next day and continued for six days. On the seventh day, the animals were sacrificed and the left testis was removed and weighed. The difference between the weights of the right and left testis in each animal served as endpoint of the assay.

Uberoi and Meyer (1967) used uterine weight of the immature rat as a measure of augmentation of pituitary gonadotropins by human chorionic gonado-tropin.

Results obtained with the *in vivo* bioassay according to Steelman and Pohley have been compared with an *in vitro* bioassay based on the estimation of estradiol produced by cultured Sertoli cells and an immunoassay by Storring et al. (1981).

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N.7.1.1.2 [^{3}H]thymidine uptake in cultured mouse ovaries

PURPOSE AND RATIONALE

Follicle-stimulating hormone increases dose-dependent the amount of [^{3}H]thymidine uptake by cultured mouse ovaries. This *in vitro* bioassay for FSH uses a tissue specific proliferation response (Ryle 1971; Boggins and Ryle 1972).

PROCEDURE

Intact ovaries are obtained from 15 day old mice. They are dissected carefully with the aid of a stereomicroscope and transferred to culture dishes. Each ovary is placed on a strip of lens tissue supported on a stainless steel mesh grid 4 mm above the floor of a plastic Petri dish and incubated in Eagle's medium supplemented with glucose and glutamine. The dishes are gassed with 5% CO_2 in air at 37 °C. Three replicate dishes are used for each concentration of the standard (0.1 and 0.4 IU/ml) and of the test preparation. [^{3}H]Thymidine (0.02 μC) is added to each dish the day after the cultures are set up. Three days later the tissue is prepared for counting. Each grid is irrigated with about 5 ml saline solution. The ovary is then transferred to a counting vial and dissolved in Soluene (Packard Instruments Comp, Inc.). Scintillation solution is added for counting in a liquid scintillation counter.

EVALUATION

Dose-response curves of uptake of [^{3}H]thymidine are established for the standard and the test preparation and potency ratios with confidence limits are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The [^{3}H]thymidine uptake by cultured mouse ovaries has found less acceptance than other *in vitro* methods described below.

MODIFICATIONS OF THE METHOD

Follicle cultures from mouse ovaries for the study of follicular metabolism have been described by Boland et al. (1993).

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N.7.1.1.3 Granulosa cell aromatase assay *in vitro*

PURPOSE AND RATIONALE

The granulosa cell aromatase assay *in vitro* is based on the principle of the *in vivo* bioassay of Steelman and Pohley, where the increase in ovarian weight is the result of FSH-induced follicular maturation and estradiol production, which further stimulates ovarian growth. The concomitant administration of HCG increases androgen production by the theca cells, thus providing androgen substrate for FSH-dependent aromatase activity in granulosa cells. Hsueh et al. (1983), Jia and Hsueh (1985) developed a sensitive *in vitro* bioassay for FSH based on the stimulation of estrogen production by cultured granulosa cells in serum free medium containing androstenedione.

PROCEDURE

Intact Sprague-Dawley rats (21–22 days old) are implanted with Silastic capsules containing diethylstilbestrol to stimulate granulosa cell proliferation. Four days after implantation, the animals are sacrificed and ovaries are dissected for granulosa cell collection. The ovaries are decapsulated, follicles are punctured with 27-gauge hypodermic needles, and granulosa cells are carefully expressed into McCoy's 5a medium. An aliquot is diluted with trypan blue stain, and viable cells are counted with a hemocytometer. Cells are cultured in 16-mm 24-well culture plates for 2–3 days at 37 °C in a humidified, 95% air-5% CO_2 incubator. Each well contains 5×10^4 viable cells in 0.5 ml McCoy's 5a medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 10^{-7} M diethylstilbestrol (DES), 10^{-6} M androstenedione (as aromatase substrate), 0.125 mM 1-methy-3-isobutylxanthine (MIX), 1 µg/ml insulin, and 30 ng/ml HCG.

For the measurement of FSH bioactivity in serum samples, each test is performed in triplicate at three dose levels (5, 10, and 20 µl). To ensure a constant volume of 20 µl serum in the total incubation volume of 500 µl, all samples are balanced with the addition of gonadotropin-free serum. For the FSH standard curve, 4% gonadotropin-free serum is added to the culture medium. In order to increase the sensitivity, the serum has to be pretreated with 12% polyethylene-glycol (PEG, mol wt 8 000). At the end of the culture period of 3 days, estradiol content in the medium is measured by radioimmunoassay.

EVALUATION

RIA data are analyzed using weighted logit-log regression analysis. Calculation of FSH bioactivity in serum samples or test preparations is performed using a standard curve fitted with a second order polynomial.

MODIFICATIONS OF THE METHOD

Beers and Strickland (1978), Wang and Leung (1983), Combarnous et al. (1984), Thakur et al. (1990) found that FSH produced a dose-dependent increase of plasminogen activator production in cultures of rat granulosa cells. This assay has been found to be extremely sensitive: as little as 10^{-15} mol of FSH could be detected.

Ax and Ryan (1979) found that FSH stimulates dose-dependently the ^{3}H-glucosamine incorporation into proteoglycans by porcine granulosa cells *in vitro*.

Bhargava et al. (1989) used normal human ovarian cells for long-term cultures. These cells produced progesterone from cholesterol or pregnenolone and estrone from androstenedione when stimulated by FSH.

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N.7.1.1.4 Sertoli cell aromatase assay *in vitro*

PURPOSE AND RATIONALE

The Sertoli cell aromatase assay was first described by Van Damme et al. (1979). This *in vitro* bioassay was developed following the observation by Dorrington et al. (1975) that FSH, but not LH, stimulates estradiol production by cultured Sertoli cells from immature rats. The assay has been further improved by Ritzén et al. (1982), Shah and Ritzén (1984), Padmanabhan et al. (1987). Generally, these assays are used to determine bioactive FSH in serum for diagnostic use, they are however also applicable to the activity of FSH preparations intended for therapy.

PROCEDURE

Seven to 10 day-old male Sprague-Dawley rats are used as a source of Sertoli cells. Testes are decapsulated, the testicular tissue is chopped twice, at right angles, with a mechanical tissue slicer and incubated in culture medium containing 0.03% collagenase and 0.003% trypsin inhibitor for 5–10 min at 34 °C. After the initial dispersal, seminiferous tubules settle to the bottom of the incubation flask, and the medium is decanted to remove interstitial cells. The tubules are washed several times; resuspended in medium that contains collagenase, trypsin inhibitor and 0.03% DNase (to prevent clumping of cells); and then incubated for 30 min at 34 °C. During incubation, dispersion of Sertoli cells is hastened by repeated aspiration with a Pasteur pipette. The resulting cell suspension is washed three times; cell number and viability are determined by counting in trypan blue dye-containing medium. Cells are suspended in a density of 5×10^5 viable cells/ml. One milliliter of cell suspension is transferred to each well of Falcon multiwell culture dishes (16 mm diameter, Falcon Plastics, Oxnard, CA).

Sertoli cells are cultured in medium comprised of the following constituents: 1 : 1 (vol/vol) mixture of Ham's F-10 nutrient mixture and Dulbecco's Modified Eagle's Medium that contains 1.2 g/liter sodium bicarbonate, 20 mg/liter gentamycine, and 1 mg/l amphotericin. Also included in the medium are: 1 µg/ml insulin, 5 µg/ml transferrin, 10 ng/ml epidermal growth factor, 20 pg/ml T_4, 10^{-8} M hydrocortisone, and 10^{-6} M retinoic acid. The cell cultures are incubated in this medium in a water-saturated atmosphere that consists of 95% air and 5% CO_2 at 37 °C.

After an initial incubation of the cell monolayers for 72 h, the medium is removed; the cells are washed once with the medium and re-incubated in the medium described above, which contains 2.5×10^{-6} M 19-hydroxyandrostenedione, FSH standard or unknown samples at various concentrations, and 0.1 mM methylisobutylxanthine (MIX). After a 24-h incubation, the medium is aspirated and centrifuged. The resultant supernatants are stored frozen until estradiol measurement by radioimmunoassay (England et al. 1974).

EVALUATION

The results are expressed as picograms of estradiol formed per ml culture medium. To evaluate changes in estradiol secretion with changes in FSH concentration, regression analyses are performed. From these data, activity ratios with confidence limits are calculated.

MODIFICATIONS OF THE METHOD

Rao and Ramachandran (1975) used isolated rat seminiferous tubule cell preparations free of Leydig cells for determination of cyclic AMP production as an *in vitro* assay for follicle stimulating hormone.

Sairam and Manjunath (1982) used seminiferous tubular suspensions of rats containing Sertoli cells, incubated them with FSH-preparations and measured cyclic AMP formation as endpoint.

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N.7.1.1.5 Receptor binding assay for FSH

PURPOSE AND RATIONALE

Significant differences between biological activity and receptor binding activity of FSH preparations have been found by Marana et al. (1979), Zaid et al. (1981), Foulds and Robertson (1983), Burgon et al. (1993). This is attributed to the assay principle of measuring binding activity, but not subsequent intracellular signaling. Several receptor binding assay procedures have been described, e.g. Cheng (1975), Andersen (1983) using bovine testes; Reichert (1976) using rat testes tubule tissue.

PROCEDURE

Membrane preparations from bovine testes are used according to the methods of Cheng (1975) and Andersen (1983). Fresh bovine testes or testes from rats weighing 220–280 g are decapsulated and rinsed with cold 0.025 M Tris-HCl buffer at pH 7.2, containing 0.3M sucrose, and then minced and homogenized with a Polytron homogenizer at maximum speed for 30 s at a concentration of 5 ml buffer per g of tissue. The homogenate is first filtered through 4 layers, and the filtrate is again filtered through 8 layers of cheesecloth. The filtrate is then centrifuged at 12 000 *g* for 30 min at 4 °C. The pellet is discarded and the supernatant is further centrifuged at 100 000 *g* for 1 h at 4 °C. The supernatant is discarded and the pellet resuspended in cold 0.025 M Tris-HCl buffer at pH 7.2, containing 10 mM $MgCl_2$, at a concentration of 1 ml buffer per g of the original weight of the testis. The isolated membranes are stored at –70 °C in aliquots of 10 ml per vial until use.

For assays, 12/75 mm glass disposable tubes are used. To each tube, 0.2 ml of 0.025 M Tris-HCl buffer at pH 7.2, containing 10 mM $MgCl_2$ and 0.1% BSA, 0.1 ml of standard FSH or unknown samples in the same buffer, 0.1 ml of ^{125}I-hFSH tracer labeled by the lactoperoxidase method (50 000 cpm, approximately 2 ng), and finally 0.1 ml of plasma membrane receptors of appropriate dilution (approximately 1–2 mg/ml) are added to reach a final volume of 500 µl per tube. All the above solutions are kept at 4 °C before use. The tubes are then shaken vigorously and incubated at room temperature for 20 h. Following incubation, the reaction is stopped by adding 3.0 ml of cold 0.025 M Tris-HCl buffer containing 0.1% BSA. After centrifugation at 4 000 rpm for 30 min, the supernatant is drained and the tip of each tube is dried. The pellet remaining at the bottom of the tube is counted in an automatic gamma counter.

EVALUATION

Specific binding (%) is defined as $(C_B - C_N) \times 100 / C_T$, where C_B is the cpm bound to the testicular receptor (pellet), C_N is the nonspecific bound cpm (nondisplacable by 1 000-fold excess of unlabeled hFSH), and C_T is the total cpm put into the tube. For standard curves, the specific binding of tracer ^{125}I-hFSH in the absence of cold hormone (6–8% by 150–200 µg of receptor protein) is taken as 100% bound ^{125}I-hFSH, and the non-specific bound ^{125}I-hFSH (1–2% of the total cpm added) as the baseline. The specific binding of ^{125}I-hFSH in the presence of a range of standard hFSH concentrations is used for calculating a standard curve for the calculation of sample concentrations.

MODIFICATIONS OF THE METHOD

Simoni, Jockenhovel and Nieschlag (1993) analyzed the biological and immunological properties of the international standard for FSH 83/575 by isoelectrofocusing and compared with other FSH preparations. The results suggested that the international standard 83/575 is not fully representative of pituitary and serum FSH, and its use for calibration of immunometric methods based on monoclonal antibodies is unlikely to resolve problems of inaccuracy in measurement of serum FSH.

Grasso et al. (1993) studied the effects of a testicular toxicant on follicle-stimulating hormone binding to membranes of cultured rat Sertoli cells.

Wakabayashi et al. (1997) reported the cDNA cloning and transient expression of a chicken gene encoding a follicle-stimulating hormone receptor.

Simoni, Weinbauer and Nieschlag (1993) analyzed the molecular heterogeneity of two batches of commercially available urofollitropin by immunofluorometric assay, radioligand receptor assay and *in vitro* bioassay.

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N.7.1.2 Luteinizing hormone (LH) = Interstitial cell stimulating hormone (ICSH)

Some of the methods described here are classical animal bioassays for LH/ICSH preparations, others are *in vitro* bioassays used in research and for clinical samples.

N.7.1.2.1 Prostate weight in hypophysectomized rats

PURPOSE AND RATIONALE

The assay is based on a biological response. Injection of ICSH into hypophysectomized male rats causes enlargement of the testes and the secondary sexual organs. The interstitial cells of the testes (Leydig cells) are stimulated by LH/ICSH and secrete androgens which in turn stimulate the accessory organs, such as the ventral prostate of the rat.

PROCEDURE

Immature male Sprague-Dawley rats at an age of 21 days are hypophysectomized. From the second to the fifth day, they receive daily subcutaneous injections of 2 or more doses of the test preparation and the LH/ICSH standard. They are sacrificed on the sixth day. Both testes and the ventral prostate are prepared and weighed. LH/ICSH – but not FSH – induces a specific dose-dependent increase of prostate weight, whereas FSH also induces an increase of testis weight. Thus, a predominant LH activity in the test material would mainly stimulate prostate weight.

EVALUATION

Dose-response curves are plotted for the of the ventral prostate in each group versus logarithm of dose of test preparation or standard allowing the calculation of activities and potency ratios with confidence limits.

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N.7.1.2.2 Superovulation in immature rats

PURPOSE AND RATIONALE

An assay for evaluation of LH and the luteinizing component of HCG was described by Zarrow et al. (1958) increasing the sensitivity of the test by priming with pregnant mare serum gonadotropin (PMSG – mainly FSH activity) and by intravenous injection of the LH test preparation.

PROCEDURE

Groups of 5 immature female rats (Charles River strain) are treated at an age of 21 days with a priming dose of 30 IU of PMS to induce follicular development followed by an intravenous injection of various doses of LH reference or test preparations 56 h later, to induce ovulation. Twenty-four hours after the final injection, the animals are sacrificed. At autopsy, the Fallopian tubes are removed and examined under a dissecting microscope at a magnification of 40×. Ovulation is easily noted by the presence of an enlarged translucent segment of the tube, through which the ova can be seen. The segment is then punctured with a dissecting needle and the entire cumulus clot containing the ova is expelled in a single mass. Due to the large number of eggs released at ovulation, the egg mass has to be treated with hyaluronidase. The number of ova is counted.

EVALUATION

Doses-response curves are obtained using three doses of standard/reference and test preparation for calculation of potency ratios with confidence limits.

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N.7.1.2.3 Ascorbic acid depletion of ovaries in PMSG/HCG primed rats

PURPOSE AND RATIONALE

Luteinizing hormone (LH = ICSH) induces dose-dependent depletion of ascorbic acid in the ovaries of rats primed with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG).

PROCEDURE

Female Sprague Dawley rats weighing 40 ±5 g are injected with 50 IU PMSG (e.g., Anteron®, Schering AG, Berlin) in 0.2 ml saline subcutaneously on day 1 at 3:00 P.M. On day 3, the rats receive 25 IU HCG (e.g., Primogonyl®, Schering AG, Berlin) subcutaneously at 9:00 A.M. On day 7, three different doses of the standard (e.g., NIH LH-S-1, National Institute of Health, Bethesda, ML) and the test substance are injected subcutaneously. Eight animals are used per group. Three hours later, the animals are sacrificed, both ovaries removed, weighed and homogenized for determination of ascorbic acid content.

EVALUATION

Dose-response curves for standard and test compound are plotted and activity ratios with confidence limits calculated.

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N.7.1.2.4 Testosterone production by Leydig cells *in vitro* induced by LH

PURPOSE AND RATIONALE

LH increases the biosynthesis and secretion of androgens in the Leydig cells of the testes. This can be used for an *in vitro* assay using isolated Leydig cells.

PROCEDURE

For Leydig cell isolation, male Wistar or Sprague-Dawley rats weighing 250–300 g are sacrificed, the testes removed and immediately decapsulated. Two testes are then digested with 7 ml of collagenase solution (1 mg collagenase/1 ml Gibco medium 199) at 37 °C for 18 min. Plastic tubes of 40 ml capacity with tight-fitting caps are used, being placed longitudinally in the water bath with constant shaking (75 cycles/min). After incubation, 15 ml of cold saline is added to each tube. The tubes are inverted a few times and then left at 4 °C for 10 min. The supernatants are then carefully siphoned off from the top. The clear supernatant is mixed with an equal volume of 26% Ficoll/0.4% BSA/medium 199 at pH 6.5, and then centrifuged at 1 500 *g* for 10 min at 4 °C. The cell pellet obtained from the centrifugation is suspended in a known volume of incubation medium (Medium 199 containing 1% BSA and 0.01% lima bean trypsin inhibitor). An aliquot of the cell suspension is taken for counting in a Coulter counter, and the balance is diluted with the incubation medium giving a density of 4×10^6 cells/ml.

For each assay, 0.25 ml of the cell suspension is used. The assay is carried out in 12×75 mm plastic tubes incubated with graded doses of test compound at 37 °C. Constant shaking (100 cycles/min) under a 95% O_2/5% CO_2 atmosphere for 3 h is required. At the end of the incubation, each tube is centrifuged, and the supernatant is assayed for testosterone by a radioimmunoassay (Dufau et al. 1972). The assay is carried out in triplicate and needs to be repeated at least once with different batches of donor animals.

EVALUATION

Dose-response curves are obtained with various doses of standard and test preparation to allow calculation of activities and potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

The effect of chemically deglycosylated human chorionic gonadotropin on cyclic AMP and testosterone production in rat Leydig cells has been used by Chen et al. (1982) for characterization.

Cultures of mouse Leydig cell tumor cells (MA10) have been used by Ascoli (1981), Whitcomb and Schneyer (1990), Dahl and Sarkissian (1993).

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N.7.1.2.5 Receptor binding assay for LH

PURPOSE AND RATIONALE

LH receptors are membrane receptors mediating the initial hormone binding step, they are suitable for structure activity studies and *ex vivo* analysis of receptor concentration changes. Luteinizing hormone is a glycoprotein composed of an α- and β-subunit. Carbohydrate moieties are attached to both. Removal of the carbohydrate chains reduces bioactivity but enhances receptor binding.

PROCEDURE

For the preparation of Leydig cell receptors, the partially purified Leydig cells obtained from Ficoll gradient centrifugation as described above for the testosterone formation test are used. The cell pellet obtained from the Ficoll gradient centrifugation is first washed twice with unlabeled radioligand buffer (0.1 M Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, 0.1 M sucrose, and 0.1% BSA). The pellet is resuspended in a known volume of the same buffer. An aliquot of this suspension is taken for cell counting in a Coulter counter, and the balance is homogenized in a Polytron homogenizer for 10 s to break up the cells. This prevents internalization of the hormone molecule during the binding assay. The iodination of LH is carried out according to the chloramine-T method (Liu et al. 1977). After addition of 2.5 ng of ^{125}I labeled LH-tracer, the binding assay is carried out at 37 °C for 2 h with constant shaking. The hormone-receptor complex is then separated by centrifugation and bound radioactivity is counted in a Packard gamma-counter.

EVALUATION

Competitive inhibition curves for specific binding are established and binding capacity is calculated.

MODIFICATIONS OF THE METHOD

Lee and Ryan (1972) described specific binding of human luteinizing hormone to homogenates of luteinized rat ovaries.

Storring and Gaines-Das (1993) described the second International Standard for Human Pituitary LH. Two new batches (coded as 80/552 and 81/535) were compared with the International Reference Preparation of Human Pituitary LH for Immunoassay (IRP 68/40) by 19 laboratories in 11 countries, using in-vivo and in-vitro bioassays, a receptor assay and immunoassays. An activity of 35 International Units of Human Pituitary LH was assigned to the contents of each ampoule coded 80/552.

Selvaraj and Moudgal (1993) used sheep luteal membranes for an LH receptor assay capable of measuring serum LH/CG in a wide variety of species.

Chen and Bahl (1993) reported a high expression of the hormone binding active extracellular domain (1-294) of rat lutropin receptor in *Escherichia coli*.

Jia et al. (1993) developed a luteinizing hormone/choriogonadotropin bioassay using 293 cells permanently transfected with the human LH receptor cDNA and a luciferase reporter gene driven by a cAMP-dependent promotor.

Selvaraj et al. (1996) established a radioreceptor assay for LH/CG in human sera using immortalized granulosa cells transfected with LH/CG receptor.

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N.7.1.3 Other gonadotropins

GENERAL CONSIDERATIONS

The pituitary gonadotropins are species specific and exist in various isoforms containing different carbohydrate moieties. For this reason, human FSH and LH are not available for therapy. For therapy of infertility, they were substituted by gonadotropins excreted in human urine and purified by extraction, hCG and hMG. Pituitary gonadotropins are now prepared by recombinant DNA synthesis for treatment of infertility.

Human chorionic gonadotropin (hCG) is excreted in the urine of pregnant women. However, hCG is also excreted by tumors, such as hydatiform mole and chorionepithelioma. The primary significance of hCG assays was for diagnosis, mainly for early diagnosis of pregnancy. HCG has predominantly a LH-like activity being useful for therapy. Purified preparations of hCG and hMG are tested by bioassays.

Human menopausal gonadotropin (hMG) is excreted in the urine from postmenopausal women and after castration. HMG has predominantly FSH-like activity and is used to induce follicular maturation.

Another gonadotropin previously used in therapy, is *pregnant mares' serum gonadotropin (PMSG).* Pregnant female horses secrete from their pituitaries high amounts of gonadotropin which is not excreted in the urine and accumulates in serum. PMSG has predominantly FSH-like activity. Purified preparations are tested by bioassays.

N.7.1.4 Human chorionic gonadotropin (hCG)

The assays described here are mostly of historical interest. For therapy, hCG is gradually replaced by recombinant human LH. It is measured as a tumor marker and widely used as a research reagent.

N.7.1.4.1 Corpus luteum formation in immature mice (Aschheim-Zondek test)

PURPOSE AND RATIONALE

Aschheim and Zodeck (1927) injected urine of pregnant women (containing FSH and LH-activity) into immature female mice and observed ovulation and formation of corpora lutea. This procedure was widely used as a biological pregnancy test and is replaced by modern *in vitro* methods. Further studies were performed by Hamburger and Pedersen-Bjergaard (1937).

PROCEDURE

Groups of 10–20 mice, 21 days of age, are treated with 5 equal subcutaneous injections of urinary extracts in the course of 48 h. The animals are sacrificed 96 h after the first injection. The ovaries are dissected and the formation of corpora lutea is observed by examination with a lens or a stereomicroscope. Rupture of follicles is indicated by blood spots.

EVALUATION

For testing hCG preparations, various doses were applied and the number of ovaries showing formation of corpora lutea were expressed as percentage dose-response curves.

The biological pregnancy test has been replaced by immunological methods available as commercial kits.

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N.7.1.4.2 Biological assay of hCG in immature male rats

PURPOSE AND RATIONALE

The LH-like activity of hCG can be determined by measuring weight increase of prostate and seminal vesicles in immature male rats.

PROCEDURE
Immature male Sprague-Dawley rats at an age between 21 and 24 days are assigned at random to six groups of at least five animals. Three doses of standard (e.g., 4, 8, and 16 IU) and corresponding doses of the test preparation are dissolved in albumin-phosphate buffer, pH 7.2, and injected subcutaneously daily over a period of 4 days. On the fifth day, the animals are sacrificed and the seminal vesicles and prostate glands are prepared and weighed.

EVALUATION
Dose-response curves of weights of seminal vesicles and ventral prostate glands are established for standard and test preparations allowing calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD
The United States Pharmacopoeia USP 23 (1995) requests standardization of hCG in immature female rats at an age of 20 to 23 days. The animals are injected subcutaneously with three different doses of test preparation or standard daily on three consecutive days. They are sacrificed on the fifth day and the uteri are prepared and weighed.

REFERENCES
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N.7.1.4.3 Receptor binding assay for hCG

PURPOSE AND RATIONALE
The hCG receptors are obtained from superovulated rat ovaries.

PROCEDURE
Ovaries from pseudopregnant rats primed with PMSG and hCG are homogenized. The homogenates are centrifuged at 2 000 *g* for 15 min and the pellets are washed 3 times with 40 mM Tris buffer and resuspended in the same buffer. The suspension is filtered through 4 layers of cheesecloth prior to use. Fresh 2 000 *g* fraction is prepared for each experiment. Purified hCG is labeled by the chloramine-T method resulting in a specific activity of approximately 60–70 μCi/μg.

For assessment of binding to receptors a mixture consisting of the 2 000 *g* fraction (equivalent to 2.5 or 5 mg of wet ovary or 45–90 μg of protein) and $0.1–30 \times 10^{-10}$ M labeled gonadotropin or other test substances are incubated in a final volume of 1 ml of 40 mM Tris buffer (pH 7.4) containing 0.1% BSA at 25 °C for 16 h. Then 1 ml ice-cold Tris buffer is added to the medium. This is immediately filtered, with suction, through Millipore EHWP filters (pore size 0.5 μm) previously wetted with 4% BSA to reduce nonspecific binding. The adsorbed material is washed with another 10 ml of ice-cold Tris buffer. The radioactivity on the filter is measured. Binding in the presence of a large excess of unlabeled hCG (200 IU/ml) is used to assess nonspecific binding.

EVALUATION
Specific binding is obtained by subtraction of the nonspecific component from total binding.

MODIFICATIONS OF THE METHOD
Selvaraj et al. (1996)described an *in vitro* bioassay and radioreceptor assay for LH/CG in human sera using immortalized granulosa cells transfected with LH/CG receptor

REFERENCES
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Selvaraj N, Dantes A, Limor R, Golander A, Amsterdam A (1996) Establishment of an *in vitro* bioassay and radioreceptor assay for LH/CG in human sera using immortalized granulosa cells transfected with LH/CG receptor. Endocrine 5:275–283

N.7.1.5 Human menopausal gonadotropin (hMG)

N.7.1.5.1 Biological assay of hMG in immature rats

PURPOSE AND RATIONALE
The predominant FSH-like activity can be measured by the increase of ovarian weight in immature female rats. The partial LH-like activity of hMG can be determined by the increase of prostate and seminal vesicle weights of immature male rats as described above.

PROCEDURE

Female Sprague-Dawley rats at an age between 21 and 24 days are assigned at random to six groups of at least five animals. Three doses of standard (e.g., a total dose of 1.5, 3, and 6 IU) and corresponding doses of the test preparation are dissolved in albumin-phosphate buffer, pH 7.2, and injected subcutaneously daily over a period of 3 days. On the fourth day, the animals are sacrificed and the ovaries are removed and weighed.

EVALUATION

Dose-response curves for FSH activity are established by mean weights of ovaries for standard and test preparation, allowing calculation of potency ratios with confidence limits.

REFERENCES

British Pharmacopoeia 1988: Biological assay of menotrophin. Follicle-stimulating activity. Appendix XIV C, pp A165–A166. London, Her Majesty's Stationary Office

N.7.1.6 Pregnant mares' serum gonadotropin (PMSG)

N.7.1.6.1 Biological assay of PMSG in immature female rats

PURPOSE AND RATIONALE

PMSG has predominantly FSH-like activity, which is determined by the increase of ovarian weight in immature female rats, as described for hMG.

REFERENCES

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N.7.1.7 Immunoassays of gonadotropins

Immunoassays of gonadotropins are important in clinical diagnosis (RIA, ELISA and similar methods). For pharmacological and biological experiments, they are measured in blood, urine and pituitary tissue, e.g. for evaluation of gonadotropin releasing hormones (as described in Sect. N.9.2.5 and N.9.2.6). Many comparisons for results of *in vivo* and *in vitro* bioassays versus immunoassays showed significant discrepancies, when expressed as the ratios of bioactive to immunoreactive LH (B/I ratio). However, there are numerous validated species-specific methods which are used for experimental endocrinology and clinical diagnosis. A few references of immunoassay methods for gonadotropins are presented.

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YoungLai EV, Yie SM, Yeo J (1992) Development patterns of bioactive and immunoreactive FSH in the female rabbit: effects of ovarectomy. Eur J Obstet Gynecol Reprod Biol 46:45–49

N.7.1.8 Gonadotropin inhibition

N.7.1.8.1 General considerations

Gonadotropin inhibition is suspected when in early testing of compounds or in toxicology studies weight reduction of gonads and the endocrine dependent organs is found. Early tests used the physiological feedback effects of endogenous steroids in rats, e.g., the parabiosis experiment . This technique in rats was applied to the relationship between central and peripheral endocrine organs, the pituitary-gonadal axis. Basically, the defi-

cit in gonadal steroid hormones after castration of one parabiotic partner induces increased secretion of gonadotropin which in turn stimulates hypertrophy of the gonads of the non-castrated partner. The procedure is described in detail by Shipley (1962). The parabiosis technique is now obsolete but has been applied again a few years ago with remarkable success to the identification of the adipose tissue hormone, leptin (see L.4.1).

REFERENCES

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N.7.1.8.2 Inhibition of gonadotropin secretion in intact animals

PURPOSE AND RATIONALE

In principle, suppression of gonadotropin secretion can be detected in the endocrine survey test, described in Sect. N.1. Young rats, e.g. 80–100 g initial body weight, are treated with gonadal steroids or their derivatives to exert feedback inhibition. Suppression of gonadal weight increase indicates gonadotropin inhibition, whereas weight increase of the sexual hormone dependent organs in castrate control groups (females or males, respectively) indicates the estrogenic or androgenic property of the test compound.

PROCEDURE

Immature male and female Sprague-Dawley rats weighing 55–65 g or young rats 80–100 g are used. Groups of 10 animals per sex and dosage group are treated daily over a period of 21 days with doses of the test compound usually by the oral or subcutaneous route. Controls receive the vehicle only. Testosterone may be used as standard in males and estradiol in females. Twenty-four hours after the last treatment, the animals are sacrificed and body weights recorded. In males, testes, seminal vesicles, ventral prostate and musculus levator ani; and in females, ovaries and uterus are dissected out and weighed.

EVALUATION

Average organ weights are calculated for each treatment group and compared with controls. The reference steroids, testosterone and estradiol, suppress gonadal weight by pituitary gonadotropin inhibition (negative feedback) and increase the weight of the sex hormone dependent organs by direct action on the tissues. Dose-response curves either pituitary inhibition or steroid action can be established for the test compounds and the standard. The relative potency of gonadotropin inhibition can be calculated.

MODIFICATIONS OF THE METHOD

As an example of a classical bioassay, gonadotropin inhibition can be tested in semicastrated male rats (Vogel 1964). Male Sprague-Dawley rats weighing 50–60 g are orchidectomized on the left side. The testis pad is weighed (without epididymis and epididymal fat). Test compounds in various doses or the standard are administered subcutaneously once daily for a period of 10 days. Controls receive the vehicle only. Standards compounds may be medroxyprogesterone acetate 0.4, 2.0, and 10.0 mg per animal and day, or 17-ethinyl-19-nor-testosterone acetate 0.1, 0.5, and 2.0 mg per animal and day subcutaneously.

On day 11, the animals are sacrificed and the testis, the adrenals, the seminal vesicles, the ventral prostate and the musculus levator ani are dissected out and weighed. Gonadotropin suppression results in atrophy of the remaining (contralateral) testis as compared with the weight of the remaining testis of the untreated control. Decrease in adrenal weight indicates feedback inhibition of the pituitary-adrenal axis. Weight of seminal vesicles, the ventral prostate and the musculus levator ani indicates androgenic activity of the test compound. Dose-response curves for the test compound and the standard and potency ratios calculated.

REFERENCES

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Vogel HG (1964) Unpublished data

N.7.1.8.3 Inhibition of ovulation and luteinization

PURPOSE AND RATIONALE

This is an assay for progestanional steroids. Progestagens exert feedback inhibition of gonadotropin secretion. Repeated administration of progestional compounds e.g. cyproterone acetate inhibit ovulation and corpus lutea formation in young female rats by reducing luteinizing hormone secretion.

PROCEDURE

Thirty-five days old female rats are treated once daily over a period of 7 days with various doses of gestagens, e.g., 0.125 to 2.0 mg progesterone or 0.025 to 0.40 mg 6α-methyl-17α-acetoxy-progesterone subcutaneously. Controls receive the vehicle only. On the 8th day, the animals are sacrificed and body weight, ovary weight and number of corpora lutea registered. Gestagens depress ovarian weight (by inhibiting follicular maturation) and inhibit corpus luteum formation.

EVALUATION

Number of rats with corpora lutea, average number of corpora lutea per animal and ovarian weight are determined. Dose-response curves can be established for these parameters.

MODIFICATION OF THE METHOD

Ovum count can be performed in the oviduct of immature mice or rats after treatment with estradiol (Austin and Bruce 1956; May 1971). Immature rats or mice are injected with various doses of estradiol or stilbestrol to initiate estrus and ovulation. Seventy-two hours later the animals are sacrificed and the oviducts are removed and examined in saline under a stereomicroscope for the presence of ova. They can be seen through the swollen translucent walls of the oviduct. The swollen part of the oviduct is then punctured with dissecting needles to release the ova, which are then counted. The results are expressed as the mean number of ova per animal. This number is dependent on the dose of estrogen. The inhibition of the estradiol response by the test compounds is determined.

Hahn et al. (1977) treated rats in diestrus with test compounds administered orally in 0.5 ml sesame oil, and again on the following day when the animals were in proestrus. The animals were sacrificed on the next day, at which they would normally have ova in the proximal segment of the Fallopian tubes. Oviducts were separately flushed with saline onto a glass microscope slide. The tubal flushings were examined with a binocular microscope, and condition and number of ova was noted.

Inhibition of ovulation by gestagens can be studied in rabbits (Shipley 1965). Progesterone is known to have a time-dependent biphasic effect on ovulation (stimulatory or inhibitory effects on secretion of pituitary gonadotropins). The timing of injection of progesterone relative to the anticipated time of ovulation is important. Thus, progesterone injected less than 4 h before the ovulation in rabbits facilitates ovulation. In contrast, ovulation is inhibited by progesterone injected four hours or longer prior to the anticipated time of ovulation (Sawyer 1952). The rabbit ovulates within a few hours after mating, after mechanical stimulation of the vagina or after an i.v. injection of copper acetate (0.3 mg/kg). Progesterone injected 24 h before induction of ovulation will prevent ovulation.

Sexually mature female rabbits weighing 3–4 kg are treated with various doses of a standard progestogen or the test compound and, 24 h later, an ovulation inducing stimulus is given. The rabbits are sacrificed and the ovaries are examined 18–24 h later. The total number of ovulation points on both of the ovaries is recorded for each animal. Dose response curves for standard and test preparations are calculated.

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Uilenbroek JTJ (1991) Hormone concentrations and ovulatory response in rats treated with antiprogestagens. J Endocrinol 129:423–429

N.7.1.8.4 Ovary-spleen-transplantation

PURPOSE AND RATIONALE

This is again a historical assay for gonadal steroids. Venous outflow from the spleen is exclusively to the liver. The steroids secreted by an autografted ovary in the spleen do not reach the peripheral circulation but are degraded to inactive metabolites in the liver. Therefore, they can not exert their systemic feed-back regulation of the pituitary, the increasing gonadotropin secretion stimulates the growth of the transplanted ovary. This effect can be antagonized by systemic application of gonadal hormones. The method has been de-

scribed by Mardones et al. (1951) for the guinea pig and by Desclin (1959) for the rat and was modified by Vogel and Jung (1962).

PROCEDURE

Female Sprague-Dawley rats weighing 100 to 120 g are anesthetized with ether. The abdominal skin is shaved and disinfected. Through a vertical incision in the skin, the abdominal wall is opened lateral to the linea alba. Both ovaries are removed. One ovary is freed from the capsule and connective tissue and weighed to the nearest 0.1 mg. With a scalpel an incision is made into the cranial end of the spleen forming a deep pocket. With a pair of forceps, the ovary is pushed into this pocket being finally placed in the middle of the spleen. The incision is closed with silk suture. The abdominal wall is closed by sutures and the skin by wound clips. The animals are treated with daily subcutaneous injection of an estrogen (e.g., 0.01, 0.1, 1.0, and 10 μg estradiol in 0.1 ml sesame oil) or a gestagen (e.g., 0.001, 0.01, 0.1, and 1.0 mg 6α-methyl-17α-acetoxyprogesterone) for a period of 5 weeks. Controls receive the vehicle only. In controls, the weight of the ovary is increased about 8-fold, i.e., from an average weight at implantation of about 10,0 mg to values around 80.0 mg. Estradiol at doses starting from 0.1 μg and 6α-methyl-17α-acetoxyprogesterone at a dose of 1.0 mg suppress ovarian growth completely. Uterus weight is increased by estradiol only.

EVALUATION

Dose-response curves or minimal effective doses of test compounds and reference standards are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The ovary-spleen-transplantation method is suitable to measure the same effects on pituitary and gonads as the parabiosis experiment, with much less stress to the animals.

REFERENCES

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Vogel HG, Jung S (1962) Unpublished data

N.7.1.8.5 Inhibition of fertility

PURPOSE AND RATIONALE

Fertility in rats can be inhibited by treatment of females with steroids inhibiting pituitary gonadotropin secretion or competing at the progesterone receptor. The test is used for contraceptive steroids.

PROCEDURE

A colony of about 100 adult female Sprague Dawley rats weighing between 200 and 250 g is established. Daily vaginal smears are taken at noon for 5 days. Fifteen regularly cycling females in proesterus are selected and caged separately. The first drug dose is administered at 3:00 P.M. (day 1). At 5:00 P.M. two vigorous males are placed with each female. On day 2, vaginal smears are taken for sperm count at 8:00 A.M. The second drug dose is administered at 4:30 P.M. On day 3, vaginal smears are taken for sperm count at 8:00 A.M. again. The 3rd drug dose is administered and sperm counts are taken at 4:30 P.M. The males are then removed. From the 4th to the 7th day the test drugs are administered in the morning. Controls receive the vehicle only. On day 9, the animals are sacrificed and the uterus examined for implantation sites.

EVALUATION

Compounds which prevent conception as evidenced by the absence of implantation sites at autopsy are given further consideration. Minimal effective doses preventing ovulation in all animals of a test group are determined.

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N.7.2 Prolactin

N.7.2.1 General considerations

The classical procedure for bioassay of prolactin is based on the work of Riddle and Bates (1939), namely, the increase of weight of the crop sacs of doves and pigeons. Other methods are based on the induction of secretory changes in the mammary glands of rodents. Most of these methods can now be replaced by cell-based assays using suitable cell les responding to prolactin.

The clinical measurements rely on immunoassays (Shiu and Friesen 1976; Jacobs 1979; Jeffcoate et al. 1986). Due to the species specificity of prolactin, a special radioimmunoassay for rats was developed.

Leroy-Martin et al. (1995) reported an immunocytochemical study of human prolactin receptors using antiidiotypic antibodies in human breast cancer.

REFERENCES

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Shiu RPC, Friesen HG (1976) Prolactin receptors. In: Blecher M (ed) Methods in Receptor Research, Part II, Marcel Dekker, Inc., New York and Basel, pp 565–598

N.7.2.2 Radioimmunoassay of rat prolactin

PURPOSE AND RATIONALE

Prolactin is a glycoprotein hormone exhibiting species specificity. For pharmacological experiments in rats, such as evaluation of gonadotropin releasing hormone activity or prolactin inhibiting factor activity, a homologous assay is necessary. The reagents for rat-prolactin and guidance for the method are provided by National Pituitary Agency, USA.

PROCEDURE

Reagents

Standard:	NIAMDD-rat-prolactin-RP-1
Antiserum:	rabbit-anti-rat-prolactin (NIAMDD-S-6)
Tracer:	^{125}I-rat-prolactin (NIAMDD-I-3)
Second antibody:	Goat-anti-rabbit-gammaglobulin (Cat. No. OTP 14/15)
Buffer:	0.01 M-phosphate-saline/0.1% bovine serum albumin, pH 7.4

Assay procedure

Standards:	0.03–1 ng/tube, 200 µl/tube
Antiserum:	1 : 4 000 100 µl/tube,
Tracer:	specific activity 250 µCi/µg, 10 000 cpm in 100 µl/tube

Standards (or sample) are incubated with antiserum for 24 h at +4 °C, the tracer is added and incubated for another 48 h. Then the second antibody (1 : 50), 200 µl/tube, is added, and incubated for 48 h at +4 °C. Separation is performed with 1.0 ml ice-cold phosphate buffered saline, pH 7.4, the vials spun at 1 300 *g* for 15 min, the supernatant decanted, and the residue counted for 1 min in a gamma-counter.

Counting equipment: gamma spectrometer.

EVALUATION

Data processing: standard curves and sample data are calculated by any suitable computer program e.g. using a spline function.

REFERENCES

Jeffcoate SL, Bacon RRA, Beastall GH, Divers MJ, Franks S, Seth J (1986) Assays for prolactin: guidelines for the provision of a clinical biochemistry service. Ann Clin Biochem 23:638–651

N.7.2.3 Pigeon crop method

PURPOSE AND RATIONALE

This is again a historical bioassay no longer applied. Riddle and Bates (1939) showed that the secretion of "crop milk" by pigeons and doves is initiated and maintained by a factor from the anterior pituitary of these birds being identical with the lactogenic hormone from mammals. Suitable assay methods (Cowie and Folley 1955) have been developed on this basis. Pigeons are exquisitely sensitive to lactogenic hormone, and the assay can be done with considerably less material than

the mammalian assays. The most sensitive assay is the so-called micromethod of local intracutaneous injections of the crop sac.

PROCEDURE
Two to 3 months old pigeons of either sex, but of uniform strain, e.g., White Carneaux, are injected intramuscularly (intrapectorally) with various doses of the test preparation or the standard once daily for 4 days. On the fifth day, the birds are sacrificed. A midventral incision is made through the skin and crop wall from keel to head. The contents of the crop and the adhering crop-milk are removed. The two lateral pouches are removed, the fat cleaned from the back of the glands and the wet weight of the glands determined. Thus, the unstimulated tissue in the dorsal midline and that around the proximal and distal opening of the crop are not weighed.

EVALUATION
Mean values of at least two doses of test preparation and standard are plotted versus logarithms of doses and potency ratios with confidence limits calculated.

MODIFICATIONS OF THE METHOD
The micromethod is based on the observation that only a small area directly over the site of injection is stimulated when lactogenic hormone is injected intradermally over the crop sac forming a "bleb" at the site of injection. A direct comparison of the potency of two different preparations can be made by a similar injection over the other crop sac. The birds are sacrificed on the fifth day and the entire crop sacs removed and examined by transmitted light (Lyons et al. 1935).

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N.7.2.4 Lactation in rabbits

PURPOSE AND RATIONALE
Only the rabbit and guinea pig have proven satisfactory for mammalian assays of lactogenic hormones. The assays are based on the prolactin induced mammary growth and milk secretion of pseudopregnant animals.

PROCEDURE
Pseudopregnancy is induced in mature estrus rabbits by intravenous injection of 50 IU of human chorionic gonadotropin. On the fourteenth day, the rabbits are examined for the presence of well-developed mammary glands characteristic of pregnancy. Various doses of the test preparation or the standard of lactogenic hormone are injected subcutaneously once daily for 6 days. On the seventh day the animals are sacrificed and the abdominal skin is incised in the midline and separated from the mammary gland underneath. The degree of enlargement of the glands with secretion is rated as follows:

–	absence of response
+	all ducts are filled with milk
++	all ducts and most of lobules are filled with milk though not greatly thickened
+++	entire gland is filled with milk
++++	mammary glands are greatly extended with milk throughout.

EVALUATION
The mean values of groups of 6 rabbits are compared with the values of the standard groups.

MODIFICATIONS OF THE METHOD
A more sensitive rabbit assay method is based on the ability of lactogenic hormone to act directly on mammary tissue (Lyons 1942). Small amounts of test compound are injected directly into one or more of the six milk ducts of a castrated, estrone-progesterone-pretreated rabbit. A localized lactation appears in the gland sector stimulated. A dose-dependent reaction was found. The assay is suitable for prolactin, placental lactogen and growth hormone.

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N.7.3 Growth hormone (GH)

N.7.3.1 General considerations

Growth hormone (GH) has been isolated from the pituitary glands of several species including man and is species specific, and for therapy has been replaced by rDNA preparations. Several methods have been described for detection and assay of growth hormone activity (Russell 1955).

Human GH consists of 190 amino acids with two disulfide bridges between cysteine residues. Human GH synthesis is controlled by two genes hGH-N and hGH-V.

GH secretion is stimulated by the growth hormone-releasing hormone (see N.9.5). GH is released in a pulsatile fashion and has a half live of about 10 min.

Receptors for GH are found in several organs, mainly in liver and muscle.

Receptor binding assays (Ilondo et al. 1991) and radioimmunoassays for growth hormone of several animal species (Greenwood et al. 1963; Peake et al. 1978) and for somatomedins (Chochinov and Daughaday 1978) have been developed.

Amit et al. (1992) measured serum growth hormone by radioimmunoassay and growth-hormone-binding protein by a binding assay with dextran-coated charcoal separation.

An immunoradiometric assay for growth hormone was described by Hofland et al. (1989).

Mertani et al. (1995) studied the cellular localization of the growth hormone receptor/binding protein in the human anterior pituitary gland.

A high performance receptor binding chromatography assay for growth hormone was described by Roswell et al. (1996).

Strasburger et al. (1996) developed an immunofunctional assay for human growth hormone. An anti-hGH monoclonal antibody recognizing binding site 2 of hGH is immobilized and used to capture hGH from the serum sample. Biotin-labeled recombinant GH-binding protein in a second incubation step forms a complex with those hGH molecular isoforms that have both binding sites for the receptor. The signal is detected after a short third incubation step with labeled streptavidin.

Functional characterization of monoclonal antibodies specific to growth hormone receptor were described by Wang et al. (1996).

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N.7.3.2 Weight gain in female rats ("growth plateaued rats")

PURPOSE AND RATIONALE

Female rats six months of age, having reached maturity, gain weight at a very slow pace; the slowing down

of the growth rate is described as "plateauing". Such rats can readily be induced to accelerate grow and weight gain by of growth hormone.

PROCEDURE

Groups of 10 adult female rats (Long-Evans or Wistar strain), 6 months old and weighing between 220 and 280 g are used. Only animals which fail to gain more than 10 g in a 20-day period are used. At least two doses of the hormone preparation and the standard dissolved in saline are injected subcutaneously daily over a period of up to 20 days. During this time, weight gains between 10 and 40 grams can be achieved. A straight line relationship exists between the logarithm of the daily dose and the growth response by body weight increase (Marx et al. 1942).

EVALUATION

The weight gain after administration of two doses of the test preparation and of the standard each are used for 2 + 2-point assays and potency ratios are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The test is needs relatively large amounts of test material. In spite of species specificity of growth hormone, rats respond to growth hormone from many other species over a limited time period, before developing neutralizing antibodies.

MODIFICATIONS OF THE TEST

Inmmature female rats hypophysectomized at 26–28 days of age can be used (Li et al. 1945; Groesbeck et al. 1987). Smaller amounts of test substance are necessary for weight gain than in adult rats.

A cell proliferation assay using a stable clone of the myeloid cell line, FDC-P1, transfected with the full length growth hormone receptor (FDC-P1-hGRH) was described by Roswell et al. (1996) as alternative to the classical hypophysectomized rat weight gain bioassay.

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N.7.3.3 Tibia test in hypophysectomized rats

PURPOSE AND RATIONALE

Hypophysectomy is followed by cessation of epiphyseal growth due to growth hormone deficiency. The width of the epiphyseal cartilage is markedly reduced after hypophysectomy. Administration of growth hormone to hypophysectomized rats induces a remarkable increase in the width of the epiphyseal cartilage plate.

PROCEDURE

Female rats of the Long-Evans or Sprague-Dawley strains are hypophysectomized at an age of 26–28 days. They are used for the bioassay 12–14 days after the operation. The increase of body weight during this period of time has to be less than 0.5 g per day indicating complete hypophysectomy. Six to 10 animals are used for each group of two doses of test preparation and standard. The solutions are administered intraperitoneally twice daily for 4 days.

On the fifth day, the animals are sacrificed, both tibiae are dissected free of soft tissue, and the bones split in half with a sharp razor at the proximal end in the mid-sagittal plane. The halves are washed in water for 10 min, immersed in acetone for 6 min, and washed again in water for 3 min. They are then placed in 2% silver nitrate solution for 2 min and rinsed with water. During the water rinse, they are exposed to a strong light which turns the calcified portions of the bone dark brown. The stained tibiae are the transferred to a microscopic stage and the width of the uncalcified cartilage plate, which does not stain and remains white, is measured under low power with a calibrated micrometer eyepiece. Ten individual readings are made across the epiphysis.

EVALUATION

Mean values are obtained from a total of 20 readings of each bone specimen. They are averaged for each dose group. With a 2 + 2-point assay, the potency ratio with confidence limits versus the standard is calculated.

CRITICAL ASSESSMENT OF THE METHOD
The test has been firmly established as a standard method for growth-promoting activity. For epiphyseal measurement, morphometry may be applied.

MODIFICATIONS OF THE METHOD
Bentham et al. (1993) described a double-staining technique for detection of growth hormone and insulin-like growth factor-1 binding to rat tibial epiphyseal chondrocytes which were incubated with biotinylated ligands with or without an excess of unlabelled ligands, followed by incubation with Vectastain ABC complex, which was then reacted with diaminobenzidine. Double staining was accomplished by carrying out the first reaction with diaminobenzidine in the presence of nickel ammonium sulphate to give a black precipitate, followed by incubation with the second ligand, then ABC complex and finally diaminobenzidine in the absence of nickel ammonium sulphate to give a brown stain.

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N.7.3.4
^{35}S uptake

PURPOSE AND RATIONALE
Cartilage contains glucosaminoglycans, e.g., chondroitinsulfate. The uptake of labeled sulfate into cartilage is greatly reduced after hypophysectomy and restored after growth hormone application. This phenomenon can be used as the basis of a bioassay of growth hormone activity (Collins and Baker 1960).

PROCEDURE
Female Sprague-Dawley rats are hypophysectomized at 21 days of age and used for experimentation three weeks later. The animals are given intraperitoneal injections of growth hormone together with radioactive labeled sulfate once daily for 4 days. Eight to 10 animals are used for at least two doses of test preparations (growth hormone derivatives) and standard. The rats are sacrificed 24 h after the last injection and the amount of radiosulfate present in the seventh rib cartilage is determined. A linear relationship exists between the uptake of radiosulfate and the hormone given over a range of 3 to 20 μg per day for 4 days.

EVALUATION
Mean values of each group and the potency of the test preparation versus standard calculated with confidence limits are calculated in a 2 + 2-point assay.

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N.7.3.5
Inhibition of glucose uptake in adipocytes *in vitro*

PURPOSE AND RATIONALE
The conversion of glucose to lipid in murine adipocytes is dose-dependently inhibited by human growth hormone (hGH). A sensitive *in vitro* bioassay was developed by Foster et al. (1993).

PROCEDURE
3T3-F442A embryonic murine fibroblasts (preadipocytes) are grown in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.1 μg/ml fungizone. The cells are plated in 60 or 100-mm plastic culture dishes at a density of 200 cells/cm^2 and grown to confluence at 37 °C under a humidified atmosphere of 90% air/10% CO_2 in medium supplemented with 25 mM glucose and 10% calf serum. Medium is replaced every 2–3 days. Once confluent, the fibroblasts are converted to cells with the characteristics of adipocytes by incubation for 48 h in medium supplemented with 25 mM glucose, 10% fetal bovine serum, 0.5 mM methylisobutylxanthine, 2 μg/ml insulin, and 250 nM dexamethasone. This medium is then replaced with medium containing 10% fetal bovine serum and 2 μg/ml insulin with changes made every 2 days for 5–8 days until at least 70% of the cells have the characteristics of adipocytes as assessed by phase contrast microscopy.

For bioassays, the medium consists of DMEM containing 5.5 mM glucose, 2% BSA, 25 nM dexamethasone, 37 nM estradiol, 10 µg/liter insulin, and 0.1 µCi/ml uniformly labeled [^{14}C]D-glucose. Cultures are co-incubated with increasing concentrations of 22 kDa human growth hormone (0.313 to 40 µg/liter) as standard or test substance or medium (controls) for 24 h. For determination of hGH in patients, 100 µl or 200 µl serum are added. After incubation, the medium is removed and discarded. The cells are treated with Doles reagent (one part heptane, four parts isopropanol, 0.1 part 1 N H_2SO_4), the plates scraped, and the contents transferred to a glass tube. Lipids are extracted by the method of Dole and Meinertz (1969), and radioactivity of the lipid is determined by scintillation counting in a liquid scintillation spectrophotometer. Results are expressed as ^{14}C counts per min/dish. Lipid accumulation in controls without hGH is taken as 100%. Logarithmic doses of 0.313 to 40 µg/l hGH result in a linear decrease of lipid accumulation. The assay is rather specific for 22 kDa hGH.

EVALUATION

From dose-response curves, activity ratios can be calculated.

MODIFICATIONS OF THE METHOD

Xu et al. (1995) studied the effects of growth hormone antagonists on 3T3-F422A preadipocyte differentiation. The antagonists not only failed to induce adipose differentiation, including late marker gene expression (adipocyte protein 2), immediate early gene expression (c-fos), and tyrosine phosphorylation of intracellular proteins, but also antagonized GH induction of c-fos expression and phosphorylation of proteins of apparent molecular mass of 95 kDa.

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N.7.3.6 Eluted stain bioassay for human growth hormone

PURPOSE AND RATIONALE

Ealey et al. (1988, 1995), Dattani et al. (1993, 1995) developed an eluted stain bioassay (ESTA for human growth hormone. This assay is based on the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium)-formazan production by quiescent Nb2 cells under influence of the hormone.

PROCEDURE

Rat Nb2 lymphoma cells are grown in suspension culture which consists of RPMI medium (Gibco) containing 50 U penicillin/ml, 50 µg streptomycin/ml, 2×10^{-3} M L-glutamine, 10% fetal calf serum and 10% horse serum. The cells are incubated in a humidified atmosphere of 5% CO_2/95% air at 37 °C.

Prior to the bioassay of hGH, the cells are transferred to a quiescent medium, which is identical in composition to the growth medium described above except that the FCS is reduced from 10% to 1%. This slows down the rate of cell division and reduces the optical density of the unstimulated control in the subsequent ESTA bioassays by about 50%. Incubation is continued in a humidified atmosphere of 5% CO_2/95% air at 37 °C for 24 h.

For the bioassay, the cells are transferred to the bioassay medium, which is the same as the growth medium but without FCS. The cells are plated out into 96-well microtiter plates, such that a final density of 2×10^5 cells/ml is obtained. Usually 50 µl of cell suspension at 4×10^4 cells/ml are added to each well. This is followed by the addition of 50 µl various concentrations of test compound or standard (0.1 to 10 mU GH/l). The cells are incubated in a humidified atmosphere of 5% CO_2/95% air at 37 °C for 96 h.

At the end of the bioassay incubation, the colorimetric endpoint is determined by the addition of 10 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) solution (5 mg/ml in phosphate buffered saline containing 0.1 mg/ml of $CaCl_2$ and $MgCl_2 \cdot 6\,H_2O$, pH 7.3) to each well: incubation is continued for 40 min at 37 °C in a dry incubator. During this time, activated cells reduce the yellow MTT salt to its purple formazan. After the 40 min incubation, 50 µl of 10% Triton X-100 in 0.1 M HCl is added to each well and the plate gently shaken for 30 min at room temperature. Bioassay responses of the 96 wells are quantified with a Biorad microtitre plate reader at optical densities at a test wavelength of 595 nm and a reference wavelength of 655 nm to correct for differential scattering.

EVALUATION

The determinations for all experiments are made on triplicate or quadruplicate microcultures and the results expressed as means and standard deviations. Dose-response curves for standard and test compounds are established.

MODIFICATIONS OR THE METHOD

The sensitivity of the assay can be increased by addition of ionic zinc (Dattani et al. 1993, 1995).

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N.7.3.7 Reverse hemolytic plaque assay for growth hormone

PURPOSE AND RATIONALE

The reverse hemolytic plaque assay as described by Neill et al. (1983), Luque et al. (1986), Smith et al. (1986) can be used for determination of growth hormone secretion by dispersed rat pituitary cells (Niimi 1994a,b).

PROCEDURE

Anterior pituitary lobes of male Sprague-Dawley rats weighing 200–250 g are collected and minced into approximately 1-mm^3 fragments. The pituitary cells are dispersed and transferred to a siliconized flask containing 3 mg/ml trypsin in Dulbecco's phosphate buffered saline containing 0.1% BSA and antibiotics, and incubated in a water bath for 5 min at 37 °C. After gentle trituration by a siliconized pipette, the cells are separated by centrifugation and rinsed with phosphate buffered saline containing 2 mg/ml DNase (type I; Sigma, St. Louis, USA). The dispersed cells are separated by centrifugation and rinsed once in phosphate buffered saline with 1.5 mg/ml trypsin inhibitor (type II-L, Sigma). The cells are washed 5 times with Dulbecco's modified Eagle's medium (DMEM) containing 0.1% BSA and resuspended. Ovine erythrocytes are coupled with staphylococcal protein A (Sigma) in the presence of 0.9% chromium chloride in normal saline. Pituitary cells (10^6 cells/ml) are combined with an equal volume of a 30% solution of protein A-coupled erythrocytes in DMEM containing 0.1% BSA and antibiotics. The cell mixture is infused into a poly-L-lysine coated Cunningham slide chamber and pre-incubated at 37 °C, 95% air/5% CO_2, for 50 min. After preincubation, the chamber is rinsed in DMEM-BSA and placed in Petri dishes containing DMEM and 10% horse serum containing 1% nonessential amino acids before performing the reverse hemolytic plaque assay.

After 24 h co-incubation, the chambers are rinsed with DMEM-BSA. Monkey anti-rat GH serum diluted 1 : 150 in assay medium is then infused into the chambers alone or with different secretagogues and incubated for 2 h. Plaque development is initiated by infusion of guinea pig complement (Gibco) at a final dilution of 1 : 40. The reaction is terminated after 0.5 h by the infusion of B-5 fixative (6 g $HgCl_2$ and 1.25 g sodium acetate in 90 ml distilled water, add 10 ml 37% formaldehyde immediately before use). The pituitary cells are stained with 0.5% toluidine blue to facilitate observation of the hemolytic plaques.

EVALUATION

In each experiment, each concentration of secretagogue or vehicle is run in duplicate, and 150–200 cells/slide are counted. Two separate experiments have to be performed. The plaque area is measured by using a calibrated ocular reticule. The area of 50 plaques/slide is measured. Statistical analysis is performed by Student's *t*-test and one-way analysis of variance.

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N.7.3.8 Determination of growth hormone isoforms by 22-kD GH exclusion assay

PURPOSE AND RATIONALE

Human growth hormone exists in variety of isoforms. In the pituitary, the most abundant isoform is 22-kD GH, while other isoforms (non-22-KD GH) are present in variable amounts. Boguszewski et al. (1966) described an analytical approach that focuses on the isoforms differing from monomeric and oligomeric 22-kD GH.

PROCEDURE

Reagents

- Recombinant 22-kD human GH (Genotropin, Pharmacia, Uppsala, Sweden),
- 22-kD GH-specific monoclonal antibody (MCB), (Genentech Inc., San Francisco, USA),
- magnetic polystyrene beads coated with rat anti-mouse IgG 1 (Dynabeads M-450), (Dynal, Oslo, Norway),
- polyclonal antibody-based IRMA (Pharmacia, Uppsala, Schweden),
- assay buffer containing phosphate-buffered saline, 5°g/l BSA, 5 ml/l Tween-20, 0.1 g/l thiomerosal.

Assay procedure

A 100-µl aliquot of serum or test solution is mixed with either 10 µl of assay buffer containing MCB (final concentration 0.3 µmol/l) or 10 µl of assay buffer without MCB. The samples are incubated for 24 h at room temperature. A 160-µl aliquot of magnetic beads coated with rat anti-mouse IgG (concentration 4×10^5 beads/µl) is added to the samples. After further incubation for 2 h at room temperature with gentle agitation in a rotator, the tubes are put in contact with a magnetic device, Dynal MPC-E (Dynal, Oslo, Norway), for 1 min. The magnetic beads with the 22-kD GH-MCB complexes ate attracted by the magnet. While the tubes are in the magnetic device, 50-µl aliquots of the supernatant are transferred to new tubes for measurement of non-22-kD GH levels in duplicate by the polyclonal antibody-based IRMA. The same procedure is performed in samples incubated with assay buffer (without addition of MCB) to determine total GH concentration.

EVALUATION

The amount of non-22-kD GH isoforms is expressed as a percentage of total GH concentration. The Mann-Whitney U test (two-tailed) is used to compare the percentage of non-22-kD GH isoforms between the groups. The method is an example for the increasing use of physicochemical methods to characterize the heterogeneity of hormone preparations obtained by rDNA methods.

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Strasburger CJ, Dattani MT (1997) New growth hormone assays: potential benefits. Acta Pediatr Suppl 412:5–11

N.7.3.9 Steroid regulation of growth hormone receptor and GH-binding protein

PURPOSE AND RATIONALE

Gabrielsson et al. (1995) studied steroid regulation of growth hormone receptor (GHR) and GH-binding protein (GHPB) messenger ribonucleic acids in the rat. GHR and GHBP, arising from alternative splicing of the same gene, show a sexually dimorphic and GH-dependent expression pattern. Multiple alternative 5'-untranslated regions are present in GHR and GHBP transcripts in the rat, one of which, GHR_1, has been shown to be liver specific and found at higher levels in females.

PROCEDURE

Animal treatment

For human GH treatment, groups of Wistar rats are implanted sc with osmotic minipumps delivering recombinant hGH at 200 µg/day for 7, 12, or 14 days. Steroid-treated animals are implanted sc with slow-release pellets delivering E_2 (25 ng/day), testosterone propionate (12.5 µg/day) or corticosterone (3.5 µg/day) for up to 2 weeks. At the end of each study, animals are sacrificed and samples of blood and liver rapidly removed.

Protein binding (GHBP) assay

Plasma GHBP levels are measured by RIA. Recombinant rat GHBP is used for iodination and reference preparation (Carmignac et al. 1992).

GH-receptor based assays

Liver samples are homogenized in 0.3 M sucrose containing 3 mM imidazole HCl, pH 7.4 (Carmignac et al. 1993). Samples of 100 µl (~2 mg protein) are incu-

bated in duplicate at 22 °C with radio-iodinated bGH (100 µl, 20 000 cpm) and 100 µl buffer (25 mM Tris-HCl, pH 7.4, containing 10 mM $CaCl_2$ and 0.1% BSA). After 2 h, 3 ml cold (4 °C) buffer are added, the tubes centrifuged at 2 700 *g* for 30 min, and the radioactivity in the pellets determined. Nonspecific binding is estimated in the presence of 1 µg unlabeled bovine GH and is subtracted from total binding to derive percent specific bGH binding per mg protein.

Generation of RNA probes

Using an *in vitro* transcription kit, probes are labeled with [^{35}S]uridine triphosphate for solution hybridization assay and [^{32}P]uridine triphosphate for Northern blots and RNase protection assays. Total GHR coding region transcripts are measured using an antisense probe corresponding to a 423-nucleotide (nt) NcoI-Kpn1 fragment (nt 989-1 411) of the λ1 clone of the rGHR inserted into the pT7T318U vector. This sequence spans the transmembrane domain and part of the intracellular domain of the GHR and is not present in GHBP mRNA. Total GHBP transcripts are determined using a 46-nt oligonucleotide probe complementary to the alternate splice sequence encoding the GHBP hydrophilic tail. This probe does not detect GHR transcripts. GHR_1-containing transcripts are measured using a probe complementary to this 5'UTR alternative exon sequence.

Northern blots

Total RNA is prepared using the method of Chomczynski and Saachi (1987). RNA is run overnight on a 2.2 M formaldehyde/3-N-morpholine propane-sulfonic acid agarose gel, transferred to a Nylon membrane and cross-linked using a Stratalinker (Strategene, La Jolla, USA). Membranes are prehybridized for 2–4 h at 60–65 °C in 50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 5 × Denhardt's solution, 50 mM Na_2HPO_4, 0.2% sodium dodecyl sulfate(SDS), 250 µg/ml salmon sperm DNA, and 100 µg transfer RNA/ml, and incubated with ^{32}P-labeled probe ($1–3 \times 10^6$ cpm/ml) at the same temperature in 50% formaldehyde, 5 × SSC, 1 × Denhardt's solution, 20 mM Na_2HPO_4, 0.2% SDS, 10% dextran, 100 µg/ml salmon sperm DNA, and 100 µg tRNA/ml. After washing (60 °C and 20 min/wash, starting at 3 × SSC/0.1% SDS and reducing to 0.3 × SSC/0.1% SDS) membranes are exposed to X-ray film with intensifying screens at –80 °C or detected by phosphorimaging.

Solution hybridization assays

Hepatic mRNAs are quantified by solution hybridization after the method of Möller et al. (1991). Liver is homogenized in 10 mM Tris-HCl, pH 7.5, containing 1% SDS and 5 mM EDTA; after proteinase K digestion. Total nucleic acids (TNAs) are extracted with phenol/chloroform, precipitated with ethanol, and the pellets dissolved in 0.2 × 10 mM Tris-HCl, pH 7.5; containing 1% SDS and 5 mM EDTA. For each assay, 10–100 µg TNAs are hybridized in duplicate with ^{35}S-labeled RNA probes (20 000–30 000 cpm) at 50 °C overnight in 40 µl, 21 mM Tris-HCl buffer, pH 7.5, containing 600 mM NaCl, 4.5 mM EDTA, 7.5 mM dithiothreitol, 0.1% SDS, and 25% formamide. Standard tubes contain known quantities of target RNA, transcribed from the sense strand of the ap-propriate plasmid and quantified by absorption at 260 nm.

After digestion with RNase A (40 µg) and RNase T_1 (2 µg) protected fragments are precipitated with 10% trichloro-acetic acid, collected by filtration (GF/C paper, Whatman), detected by scintillation counting, and expressed as specific mRNA levels (attomoles per µg TNA).

RNase protection assay

Total RNA (20 µg) is hybridized under the same conditions as above, except that a ^{32}P-labeled probe (500 000 cpm) is used. After overnight incubation at 50 °C, the samples are treated with 300 µl of a solution of 10 mM Tris, pH 7.5, 5 MM EDTA, and 300 mM NaCl, containing RNase A (40 µg/ml) and RNase T_1 (2 µg/ml), for 30 min at room temperature. After incubation with 50 µg proteinase K and 20 µl 10% SDS for 15 min at 37 °C, samples are extracted with phenol/chloroform and ethanol precipitated with 10 µg tRNA as carrier. The pellets are dissolved in gel-loading buffer (10 mM EDTA and 1 mg/ml bromophenol blue in 80% formamide), denatured at 85 °C for 4 min, and run on an 8 M urea/6% polyacrylamide gel. Detection is by autoradiography or phosphorimaging.

EVALUATION

Data are calculated as mean ±SEM. Differences between treatment groups are assessed using Student's *t*-test, or analysis of variance followed by Student-Newman-Keul's or Dunnett's tests. A difference of $p < 0.05$ is considered as significant.

MODIFICATIONS OF THE METHOD

Nilsson et al. (1995) described the expression of functional growth hormone receptors in cultured human osteoblast-like cells.

To measure the absolute number of mRNA molecules encoding the growth hormone receptor in human tissue, Martini et al. (1995) developed a quantitative polymerase chain reaction assay.

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N.7.4 Adrenocorticotropin (ACTH)

N.7.4.1 Adrenal ascorbic acid depletion

PURPOSE AND RATIONALE

The administration of pituitary adrenocorticotropic hormone (ACTH) is followed by a decrease in the amount of ascorbic acid present in the adrenals. The depletion of adrenal ascorbic acid is a function of the dose of ACTH administered. This relationship has been used for a quantitative assay of ACTH by Sayers et al. (1948). The method has been selected for standardization of ACTH by several Pharmacopoeias, e.g. The United States Pharmacopeia USP 23 (1995), Deutsches Arzneibuch 1986 and British Pharmacopoeia 1988. Furthermore, the test has been used for evaluation of synthetic corticotropin analogues (Geiger et al. 1964; Vogel 1965, 1969a and b). A similar test is used for luteinizing hormone action on the rat ovary.

PROCEDURE

Male Wistar rats weighing between 100 and 200 g are hypophysectomized one day prior to the test. The range of weights in any one test should not exceed 15 g. For one test with 3 doses of test preparation and standard each, at least 36, preferably 60, hypophysectomized rats are necessary.

Solutions

Five units of the International Standard for corticotropin (Bangham 1962) or an amount of test preparation supposed to contain about 5 units are dissolved in 0.25 ml 0.5% phenol solution and diluted with 8.1 ml 15% gelatin solution. In this way, 0.5 ml contain 300 mU ACTH. Three ml of this solution are diluted with 6.0 ml gelatin solution (to prevent adsorption to glassware), resulting in a content of 100 mU ACTH per 0.5 ml. Three ml of this solution are again diluted with 6.0 ml gelatin solution, resulting in a content of 33 mU ACTH per 0.5 ml.

The hypophysectomized rats are randomly distributed to 6 groups. Each rat receives subcutaneously 0.5 ml of one of the various concentrations of test preparation or standard. Three hours after injection, the animals are anesthetized, both adrenals removed, freed from extraneous tissue and weighed. The rats are sacrificed and the scull opened to verify completeness of hypophysectomy.

The adrenals are homogenized in 4% trichloroacetic acid and the ascorbic acid determined according to the method of Roe and Kuether (1943). Other methods have been described.

ASCORBIC ACID DETERMINATION

Reagents

One hundred mg L-ascorbic acid are dissolved in 100 ml 4% trichloroacetic acid. Twenty ml of this solution are diluted with 4% trichloroacetic acid to achieve a 0.2% ascorbic acid solution and 2 ml of this solution are diluted with 4% trichloroacetic acid to achieve a 0.02% ascorbic acid solution.

Sulfuric acid (85%) is obtained by adding 900 ml concentrated sulfuric acid to 100 ml distilled water.

Two g dinitrophenylhydrazine are dissolved in 100 ml 9 N H_2SO_4 (75 ml distilled water and 25 ml concentrated sulfuric acid).

Six g thiourea are dissolved in 100 ml distilled water.

Calibration

Trichloroacetic acid (4%) is added to 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0 ml of the 0.02% ascorbic acid solution and 1.0, 1,5 and 2.0 ml of the 0.2% ascorbic acid solution to reach a final volume of 8.0 ml. Hundred mg charcoal is added to each sample and thoroughly mixed by shaking for 1 min. After 5 min the solutions are filtered. An aliquot of 0.1 ml of the 6% thiourea solution is added to 2.0 ml of the filtrate followed by 0.5 ml dinitrophenylhydrazine solution. The mixture is shaken and heated for 45 min at 57 °C in a water bath. The solutions are placed in an ice-cold water bath

and with further cooling 2.5 ml of the 85% sulfuric acid are added. The calibration curve is established at a wave length of 540 µm using the solutions without ascorbic acid as blank.

Preparation of the adrenals

Both adrenals are homogenized in glass tubes containing 200 mg purified sand and 8.0 ml 4% trichloroacetic acid. The reagents are added as described for the calibration curve.

EVALUATION

The potency ratio including confidence limits is calculated with the 3 + 3 point assay.

MODIFICATIONS OF THE METHOD

The original method, as described by Sayers et al. (1948), uses intravenous administration of ACTH and the difference of ascorbic acid in the left adrenal before injection and the right adrenal one hour after injection as endpoint. Different values of activity of synthetic peptides versus the international standard resulted from different ways of administration (Vogel 1965).

The ascorbic acid depletion test can also be performed in dexamethasone-blocked rats. However, different potency ratios of synthetic corticotropin analogues have been found than in hypophysectomized rats (Vogel 1969a). The difference most likely depends on the dexamethasone blocking dose.

Other authors, including British Pharmacopoeia and Deutsches Arzneibuch, use the 2,6-dichloro-phenol-indophenol method for determination of ascorbic acid.

The glands are homogenized in 2.5% metaphosphoric acid with the addition of a small quantity of washed sand. With additional 2.5% metaphosphoric acid a final volume of 10 ml is reached. Five ml of the filtrate are added to 5 ml indophenol acetate solution, and the absorbance of the mixture is read immediately in a photometer with a 520 mµ filter. The indophenol acetate solution is prepared by dissolving 15 mg 2,6-dichlorophenol-indophenol in 500 ml distilled water and dissolving 22.65 g sodium acetate $\cdot 3\,H_2O$ in 500 ml distilled water and mixing equal volumes.

A cytochemical bioassay of corticotropin was described by Chayen et al. (1976).

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N.7.4.2 Corticosterone blood levels in dexamethasone blocked rats

PURPOSE AND RATIONALE

Corticotropin activity can be measured by the increase of corticosterone in venous blood of hypophysectomized or dexamethasone-blocked rats. The test can be used to measure time-response curves of corticotropin analogues or depot-preparations (Vogel 1969a,b). The sensitivity can be increased by determining corticosterone in adrenal venous blood after cannulation of the adrenal vein (Retienne et al. 1962).

PROCEDURE

Male Sprague-Dawley rats weighing 150–200 are injected subcutaneously 24 h and 1 h prior to subcutaneous injection of the ACTH-preparation or the standard with 5 mg/kg dexamethasone in oily solution. Eight rats are used for each dose of test preparation or standard.

Various time intervals after ACTH injection, the rats are anesthetized with 60 mg/kg pentobarbital i.p. and blood is withdrawn by cardiac puncture. One ml plasma is diluted with 2 ml distilled water and extracted (washed) with 5 ml petrolether to remove the lipids. The petrol ether is discarded. Two ml of the water layer are extracted twice with 5 ml methylene chloride by vigorous shaking for 15 min. The methylene chloride phase is separated by centrifugation. Both methylene chloride extracts are unified and shaken with 1 ml ice-cold 0.1 N NaOH. The water phase is immediately removed and the methylene chloride extracts dried by addition of dry sodium sulfate. An aliquot of 5 ml of the methylene chloride extract is mixed with 5 ml of the fluorescence reagent (7 parts concentrated sulfuric acid, 3 parts 96% ethanol, v/v). After vigorous shaking, the methylene chloride phase is removed and fluorescence is measured with primary filters of 436 mμ and secondary filters of 530–545 mμ. For calibration, concentrations of 0, 20, 50, 100, and 250 μg/ml corticosterone are treated identically and measured in each assay.

EVALUATION

Using 3 doses of test compound and standard, activity ratios with confidence limits can be determined after each time interval with the 3 + 3 point assay giving evidence for the duration of action (Vogel 1969a,b).

MODIFICATIONS OF THE METHOD

Pekkarinen (1965) used fluorometric corticosteroid determinations in guinea pigs resulting in highly deviating activity ratios of synthetic and commercial corticotropins as compared with the international working standard of ACTH.

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N.7.4.3 *In vitro* corticosteroid release

PURPOSE AND RATIONALE

An *in vitro* assay of corticotropin was described by Saffran and Schally (1955). This test has been modified by Van der Vies (1957) and used by several authors (Staehelin et al. 1965; Vogel 1969).

PROCEDURE

Solutions

"Double Ringer" solution is prepared as follows:

4.50%	(w/v) sodium chloride	200.0 ml
5.75%	potassium chloride	8.0 ml
6.10%	calcium chloride	6.0 ml
10.55%	monopotassium phosphate	2.0 ml
19.10%	magnesium sulphate	2.0 ml
	double distilled water up to	545.0 ml

Final solution:

54.0	ml	double Ringer
26.0	ml	1% glucose solution (freshly prepared)
29.0	ml	double distilled water
21.0	ml	1.3% $NaHCO_3$ solution which has been gassed with carbon dioxide at room temperature for 1 h.

The final solution is gassed with a mixture of 95% O_2 and 5% CO_2 for 10 min.

Preparation of adrenals

Twenty male Sprague-Dawley rats weighing 150–200 g are anesthetized with 50 mg/kg pentobarbital sodium i.p. applying as little handling stress as possible. The adrenals are removed and freed of connective tissue taking care that the adrenals are not damaged. Each adrenal is carefully cut into 4 quarters with fine scissors. The 8 quarters of each rat are randomly distributed to 20 preweighed incubation vessels filled with 1.5 ml of final solution. The flasks are mounted on

Warburg manometers or placed into a suitable shaking water bath and are gassed under continuous shaking for 1 h with a mixture of 95% O_2 and 5% CO_2 at 38 °C (= preincubation period).

The flasks are removed from the bath at the end of the preincubation period. The medium is aspirated as much as possible by means of a small tube attached via a collection bottle to the vacuum line. An aliquot of 1.4 ml fresh medium is added to each flask.

To 5 vessels each of the following solutions are added:

10.0 mU/0.1 ml ACTH standard
50.0 mU/0.1 ml ACTH standard
10.0 mU/0.1 ml test preparation
50.0 mU/0.1 ml test preparation
50.0 mU/0.1 ml medium (control)

The vessels are again incubated and gassed with a mixture of 95% O_2 and 5% CO_2 at 38 °C under continuous shaking for 2 h.

One ml aliquots of the medium of each vessel are transferred to carefully cleaned glass-stoppered tubes containing 2 ml methylene chloride. The tubes are vigorously shaken for 1 min and centrifuged for 5 min. The methylene chloride phase is transferred with a long needle and a syringe to a quartz micro-cuvette and readings are taken at 225, 240, and 255 mμ.

Dry weight of the adrenals is determined by heating the incubations vessels to 150 °C for 2 h.

EVALUATION

Extinction values are calculated for the maximum of absorption (Allen 1950) according to the formula:

$$E = E_{240} - \frac{E_{225} + E_{255}}{2}$$

The potency ratios are calculated with the 2 + 2-point assay.

MODIFICATIONS OF THE METHOD

Saffran et al. (1971) described a flow-through system for the study of adrenocortical function by rat tissue *in vitro*, in which the fluorometric measure of corticosterone is completely automated.

Corticosterone is now conveniently determined by RIA or HPLC.

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N.7.4.4 Thymus involution

PURPOSE AND RATIONALE

This is a classical indirect bioassay based on the steroid response of the target organ and its effect. Administration of corticotropin decreases thymus weight (due to corticosterone secretion) and induces an increase of adrenal weight (Hayashida and Li 1952; Thompson and Fisher 1953). Young rats respond with an involution of the thymus gland to graded doses of corticotropin (Rerup 1958). A similar assay is used for adrenal steroids.

PROCEDURE

Sprague-Dawley or Wistar rats of either sex, 7–10 days of age, weighing 10–15 g are used. Littermates are preferred. The animals are distributed at random to three groups of the standard and 3–5 groups of the unknown sample. The ACTH standard or test com-

pounds are injected at different doses once daily subcutaneously for 3 days. Twenty-four hours after the last injection, the animals are sacrificed, the thymus dissected out and weighed to the nearest 0.1 mg. The response is expressed as the average of the individual values for each dose level.

EVALUATION

Dose-response curves are established and potency ratios calculated using a 3 + 3 point assay.

MODIFICATIONS OF THE METHOD

The sensitivity of the assay can be increased using the quotient between increase of the weight of the adrenals and decrease of the weight of the thymus gland (Hohlweg et al. 1960). Male Wistar rats at an age of about 3 weeks weighing 18–22 g are injected three times daily in intervals of 4 h during three days with the test preparation or the standard dissolved in 10% gelatine solution. The animals are sacrificed 18 h after the last injection and the adrenals and the thymus gland removed and weighed. The ratio of adrenal weight to thymus weight provides a steep dose-response curve.

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N.7.4.5 Receptor binding assay for ACTH

PURPOSE AND RATIONALE

As for other hormones, receptors have been used as the test preparations for comparison of the binding affinities of adrenocorticotropin peptides using a cloned mouse adrenocorticotropin receptor expressed in a stably transfected HeLa cell line (Kapas et al. 1996). Such assays measure the initial membrane binding but not strictly the biological activation e.g. of adrenal cells or melanocytes.

PROCEDURE

Hela cells are seeded into 12-well culture plates at a density of 10^6 cells/well. On the second day of culture, the cells are washed as follows: 2× in 1 ml of ice-cold 0.9% NaCl, 1× in 1 ml of ice-cold glycine (50 mM glycine, 100 mM NaCl, pH 3.0) for 5 min, 2× in 0.5 ml of ice-cold 0.9% NaCl. Cells are then incubated for 60 min at 20 °C with increasing concentrations of nonradioactive ACTH or various ACTH analogues and the reactions initiated on the addition of [^{125}I-iodotyrosyl^{23}]ACTH[1-39] (2 000 Ci/mmol; final concentration 0.1 pmol/liter) in DMEM. At the end of the incubation, the medium is removed and the cells washed three times with 0.9% NaCl and then dissolved in 0.5 M NaOH/0.4% sodium deoxycholate. Each point is determined in triplicate, and the radioactivity is measured using a gamma-counter. Specific binding is determined by subtracting from the total binding the radioactivity associated with cells in the presence of 10^{-5} M nonradioactive ACTH.

EVALUATION

Binding parameters are determined using a computer assisted calculation, e.g. the LIGAND program (Munson and Rodbard 1980).

MODIFICATIONS OF THE METHOD

Penhoat et al. (1993) reported the identification and characterization of corticotropin receptors in bovine and human adrenals by covalent cross-linking of radiolabeled ACTH with the bifunctional cross-linking agent disuccinimidyl suberate to cultured bovine adrenal fasciculata reticular cells and to crude plasma membrane fractions prepared from both human and bovine adrenals.

Lebrethon et al. (1994), Penhoat et al. (1995) studied the regulation of ACTH receptor mRNA and binding sites by ACTH and angiotensin II in cultured human and bovine adrenal fasciculata cells.

Picard-Hagen et al. (1997) found, that glucocorticoids enhance corticotropin receptor mRNA levels in ovine adrenocortical cells.

Zavyalov et al. (1995) described receptor binding properties of peptides corresponding to the ACTH-like sequence of human pro-Interleukin-1_α.

Naville et al. (1996, 1997) developed a stable expression model in order to characterize the human ACTH receptor by binding studies and functional coupling to adenylate cyclase.

Schioth et al. (1996) described the pharmacological distinction of the ACTH receptor from other melanocortin receptors in the mouse adrenocortical cell line Y1.

Moreover, melanocortin receptors do not have a binding epitope for ACTH beyond the sequence of alpha-MSH (Schioth et al. 1997).

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N.7.5 Thyrotropin (TSH)

N.7.5.1 General considerations

The thyroid stimulating hormone TSH can be determined by bioassays and immunoassays, as with other polypeptide hormones (Utiger 1979; Spencer 1994; Meinhold et al. 1994).

Large scale synthesis of recombinant human thyrotropin has been reported (Cole et al. 1993; Hussain et al. 1996). The role of the thyrotropin receptor has been reviewed by Vassart and Dumont (1992). Castagiola et al. (1992) described a binding assay for thyrotropin receptor autoantibodies using the recombinant receptor protein. A brain-derived TSH receptor has been cloned and expressed (Bockmann et al. 1997), on accordance with the developmental effects of TSH in the fetus and newborn. Binding characteristics of antibodies to the TSH receptor were described by Oda et al. (1998).

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N.7.5.2 Thyroid histology

PURPOSE AND RATIONALE

Hypophysectomy results in atrophy of the thyroid gland which is reversed by administration of thyrotropin. In the thyroid of normal young guinea pigs (Junkmann and Schoeller 1932; McGinty and McCullough 1936) or chicks (Jones 1939) characteristic histological changes are observed after administration of thyrotropin, associated with an increase of thyroid weight (goitrogenic response). In classical bioassays, these findings were the basis for standardizing by biological units.

PROCEDURE

Male guinea pigs weighing 180–200 g are injected once daily on 4 successive days. Thyroids are removed on the sixth day, weighed, and embedded for histological examination. Administration of thyrotropic hormone is followed by colloid resorption, increased vascularity, and increased epithelial cell height. Several regions of the thyroid are examined histologically. Alternatively, computer-assisted morphometry may be used. Rating scores are defined between +1 and +4.

EVALUATION

The rating scores are averaged and compared between test preparation and standard.

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N.7.5.3 Iodine uptake

PURPOSE AND RATIONALE

This is a bioassay for thyroid activation related to the initial step of TSH action. The uptake of iodine (trapping) as well as the release of the newly formed iodinated thyroid hormones is under control of thyrotropin (and in animal assays, under control of hypothalamic TRH). As a consequence, the uptake of ^{131}I and the release of ^{131}I-labeled thyroxin are increased after administration of TSH. A method using ^{131}I-release in mice has been described by McKenzie (1958) and modified by Sakiz and Guillemin (1964).

PROCEDURE

Female mice weighing 10–15 g are kept in a temperature-controlled room and fed a low iodine diet for 10 days. They are then injected intraperitoneally with 1.5 μC ^{131}I, followed 5 h later by 10 μg L-T_4 subcutaneously. Twenty-four hours later they receive a second injection of 5 μg L-T_4 and are used 48 h after the last injection. Under ether anesthesia 0.25 ml blood is withdrawn from the jugular vein in a heparinized syringe. Various doses of the test preparation or standard TSH are injected by the same route in 0.3-ml volume. Two hours later, again under ether anesthesia, a second 0.25-ml sample is taken, and the radioactivity measured. The increase of radioactivity in the blood samples is dependent on the dose of TSH.

EVALUATION

A 4-point assay technique is used with 6 observations for each of 2 doses of the standard and of the unknown preparation.

MODIFICATIONS OF THE METHOD

Depletion of ^{131}I from the thyroids of chicken was used as endpoint for a TSH assay by Bates and Cornfield (1957).

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N.7.5.4 TSH bioassay based on cAMP accumulation in CHO cells

PURPOSE AND RATIONALE

Indirect cell assays measure the activation of protein kinase A (PKA) through adenylcyclase. Several cell assays for thyrotropin using cultured FRTL-5 cells were described (Vitti et al. 1986; Nissim et al. 1987; Horimoto et al. 1989). Persani et al. (1993) reported a cell assay for human thyrotropin using measurement of cAMP accumulation on Chinese hamster ovary cells transfected with the recombinant TSH receptor.

PROCEDURE

Cells of the CHO-R strain JP-09 are cultured in Petri dishes in RPMI-1 640 medium supplemented with

1 mM glutamine and 10% fetal calf serum. In these cells TSH biological activity is evaluated by measuring cAMP production. Cells are harvested from Petri dishes using a Trypsin-EGTA mixture and seeded in 96-well plates (10 000 cells/well). Cells are fed with fresh RPMI-1 640 medium 24 h after seeding. The assay is run after 48 h. After washing, 100 µl of TSH standard or samples diluted in hypotonic or isotonic medium containing 0.4% BSA, 10 mM HEPES and 0.5 mM isobutylmethylxanthine, are incubated for 2 h at 37 °C. Three different dilutions of immunoconcentrated TSH are bioassayed in triplicate, as are TSH preparations. CAMP is measured in non-acetylated samples by an RIA method using a commercial polyclonal anti-cAMP antibody (Vitti et al. 1986).

EVALUATION

Dose response curves or single point comparisons are used for potency or activity estimates.

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N.7.6 Hormones related to TSH

N.7.6.1 General considerations

These hormonal factors are now characterized by molecular cloning. One of the characteristic symptoms of thyroid activation (Graves or Basedow disease) is exophthalmus. A dissociation of the exophthalmus producing activity from the TSH activity has been found (Dobyns and Steelman 1953). Moreover, time-response curves for TSH activity from serum of patients with hyperthyroidism differed from those of the standard TSH, giving evidence for an abnormal factor, the long acting thyroid stimulating factor (LATS). Bioassays for these factors have been developed. TSH receptor stimulating antibodies have also been identified.

N.7.6.2 Assay of exophthalmus-producing substance (EPS) in fishes

PURPOSE AND RATIONALE

Some TSH fractions were reported to produce more exophthalmus than others (Dobyns and Steelman 1953). A second fraction containing exophthalmus producing substance (EPS) could be separated (Brunish et al. 1962). The activity of this substance was demonstrated in fishes.

PROCEDURE

Fundus heteroclitus Linn., the common Atlantic minnow, has been found to be the suitable animal model (Albert 1945; Sobonya and Dobyns 1967). Other fish species, such as Carassius auratus, the common gold fish (Haynie et al. 1962) and Cyprinus carpio (der Kinderen et al. 1960) were also used but gave less reliable results (Sobonya and Dobyns 1967). *Fundus heteroclitus* Linn., the common Atlantic minnows, 8–10 cm in length, are kept in a tank of running tap water at 10 °C in winter and 18 °C in summer. Before treatment the intercorneal distance is measured with Vernier calipers to the nearest of 0.1 mm. The test compound and the standard are injected intraperitoneally at various, but at least two, doses. To avoid loss of fluids by the intraperitoneal injection route, the needle is inserted into the cloaca, through the rectum, over the pelvic girdle, and into the peritoneal cavity for a distance of about 1.2 cm. The volume of fluid injected should be 0.1–0.5 ml. The effect of the hormone is to cause protrusion of the eyeballs. The intercorneal distance is measured again after 24, 48, and 72 h. The increase in proptosis is expressed as percentage of the intercorneal distance found at the beginning of the test.

EVALUATION

The increases of intercorneal distance after each dose are averaged and activity ratios with confidence limits calculated from the 2 + 2-points assay.

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N.7.6.3 Assay of long acting thyroid stimulating factor (LATS) in mice

PURPOSE AND RATIONALE

This is a modified TSH assay. In the assay of McKenzie (1958) (see N.7.5.3) mice previously injected with ^{131}I show a maximum increase in serum ^{131}I after an interval of 2–3 h when TSH is administered. In the assay of serum from thyrotoxic patients, Adams (1958) noted that the maximum response in guinea pigs did not occur until after 16 h. The abnormal responses to thyrotoxicosis sera suggested the presence of an additional factor different from TSH.

PROCEDURE

Mice maintained on a low iodine diet for 10 days are injected with 15 µC ^{125}I and 10 µg Na L-T_4. Four days later, 0.1 ml of blood is obtained by retroorbital puncture immediately before the injection of the test substance and 2 and 9 h later. Radioactivity in the blood is then measured. By definition, radioactivity which is maximal after 2 h is indicative of TSH; whereas long acting thyroid stimulator LATS causes a maximal increase at 9 h.

EVALUATION

The increases of radioactivity after 2 and 9 h are compared and evaluated by statistical methods.

MODIFICATIONS OF THE ASSAY

Ikeda and Nagataki (1983), Ikeda et al. (1984) used male DDY mice weighing 15 g. They were fed a low iodine diet for 14 days and then injected daily with 1 µg of 3,5,3'-triiodothyronine (T_3) s.c. and given T_3 (5 µg/ml) ad libitum in drinking water until sacrifice. From the fifth day of T_3 treatment they were injected i.p. with 0.25 ml of LATS-positive serum for 9 days. Groups of 5 mice were sacrificed before, 1, 3, 5, 7 and 9 days after the first injection of LATS. One µCi (0.5 ml) of Na [^{131}I] was administered i.p. 1 h before sacrifice. Thyroid lobes were excised, weighed and radioactivity was measured by a gamma counter. Immediately before injection of Na [^{131}I], approximately 60 µl of blood were collected from the orbital plexus with heparinized capillary tubes and centrifuged at 12 000 r.p.m. for 3 min. T_4 concentrations in serum were determined by radioimmunoassay.

Ealey et al. (1984, 1985) developed a sensitive cytochemical bioassay for thyroid stimulators, using reference preparations of thyrotropin and long acting thyroid stimulator. Thyroid stimulators cause changes in lysosomal membrane permeability within the thyroid follicular cells of guinea pigs, which can be monitored by measuring increased intralysosomal enzyme activity (in the case of this assay, naphthylamidase), with a chromogenic substrate, leucine-2-naphthylamide, which itself does not readily permeate the lysosomal membrane in unstimulated cells.

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N.8
Posterior pituitary hormones

N.8.0.1
General considerations

Oxytocin and vasopressin were the first hormones isolated from the pituitary (Dale and Laidlaw 1912; Hogben et al. 1924; Fromherz 1926; Schaumann 1937). Vasopressin has hypertensive and antidiuretic activity, as well as endocrine functions (Hedge and Huffman 1987) and effects on the central nervous system (Gash et al. 1987). In terms of bioassays however, the antidiuretic activity is the main parameter. Many analogues of vasopressin have been synthesized resulting in selective agonists and antagonists (Vogel and Hergott 1963; Manning et al. 1987; Mah and Hofbauer 1987; Allison et al. 1987). Different types of vasopressin receptors have been identified: V_1 (V_{1a}, V_{1b}) and V_2-receptors (Jard et al. 1976; Jard et al. 1986; Fahrenholz et al. 1988; Walker et al. 1988; Burnatowska-Hledin and Spielman 1989).

Oxytocin receptors have been described in several organs, such as uterus, mammary gland and CNS (Soloff 1976; Hruby et al. 1990).

Synthesis and development of several orally active, nonpeptide oxytocin antagonists were reported (Bell et al. 1998; Kuo et al. 1998).

Radioimmunoassays are available for both oxytocin (Kagan and Glick 1978) and vasopressin (Glick and Kagan 1978).

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N.8.1 Oxytocin

N.8.1.1 Isolated uterus

PURPOSE AND RATIONALE

Several authors, such as Dale and Laidlaw (1912), Fromherz (1926), Glaubach and Molitor (1932), Lipschitz and Klar (1933), Simon (1933) used the isolated uterus of virgin guinea pigs as a sensitive test for determination of oxytocin activity. The isolated uterus of the rat (Holton 1948) is less sensitive but in contrast to the guinea pig, the rat uterus shows no spontaneous contractions in solutions with low calcium and glucose concentrations. Historically, the method has been adopted by several pharmacopoeias, e.g., by the British Pharmacopoeia (1988). The United States Pharmacopeia 23 (1995) uses the isolated guinea pig uterus for determination of oxytocin activity in vasopressin preparations. Physicochemical assays are now used for standardising drug content, instead of the biological responses.

PROCEDURE

Female Sprague-Dawley or Wistar rats weighing 120–200 g are used. Eighteen to 20 h prior to the assay, the rat is injected i.m. with 100 µg of estradiol benzoate for priming (receptor induction). Immediately before the assay the rat is tested for estrogen induced epithelial proliferation by vaginal smear. One horn of the uterus is suspended in an organ bath containing a solution of the following composition:

Sodium chloride	6.62 g/l
Potassium chloride	0.45 g/l
Calcium chloride	0.07 g/l
Sodium hydrogen carbonate	2.56 g/l
Disodium hydrogen orthophosphate	0.29 g/l
Sodium dihydrogen orthophosphate	0.03 g/l
Magnesium chloride	0.10 g/l
D-glucose	0.50 g/l

The bath temperature is maintained at 32 °C, a temperature at which spontaneous contractions of the uterus are abolished and the preparation maintains its sensitivity. The solution is bubbled with a mixture of 95% O_2 and 5% CO_2. The preparation is loaded with 1–2 g and the contractions recorded using a Statham transducer and a polygraph. Two doses of the standard preparation are added ranging usually between 10 and 50 microunits per ml organ bath. The preparation examined is diluted in such a way to obtain responses on the addition of two doses similar to those obtained with the standard preparation. The ratio between the two doses of the preparation being examined should be the same as that between the two doses of the standard preparation. The two doses of the standard preparation and the two doses of the preparation being examined are given according to a randomized block or a Latin square design and at least six to eight responses to each are recorded. The doses should be recorded at regular intervals of 3 to 5 min depending on the rate of recovery of the muscle.

EVALUATION

The activity and potency ratios with confidence limits are calculated from the 2 + 2-points assay.

MODIFICATIONS OF THE METHOD

The isolated rat uterus is also used to test spasmolytic activity of various drugs against oxytocin as spasmogen. Liebmann et al. (1993) used the rat uterus to test the pharmacological and molecular actions of the bradykinin B_2 receptor antagonist, Hoe 140.

The method described by Schübel and Gehlen (1933) using the **uterus of cats** two to 4 days after partum is of historical interest only.

In addition to the isolated rat uterus, Berde et al. (1957) used the **rat uterus *in situ***, the **cat uterus *in vitro***, and the **cat uterus *in situ*** for evaluation of synthetic analogues of oxytocin.

Murray and Miller (1960) observed characteristic postural changes in rats following administration of oxytocin to unanesthetized rats described as "cramping" which was dose-dependent in estrogen-pretreated animals.

An *in vitro* **hen oxytocic assay** was designed by Munsick et al. (1966). Muscle strips of the uterine portion of the oviduct of laying hens are dissected and suspended in a van Dyke-Hastings solution containing 0.15 mM/l calcium, 0.5 mM/l magnesium and 100 mg% glucose. The strips are 2–3 cm long and 2–3 mm wide. The solution is gassed with oxygen containing 5% carbon dioxide and maintained at a temperature of 43 °C. These conditions are necessary to prevent spontaneous contractions.

The **isolated uterus from immature guinea pigs** was used for evaluation of oxytocin activity by Fromherz (1926) and by Vogel and Hergott (1963).

Guissani et al. (1995), Pettibone et al. (1996) reported the effect of oxytocin antagonists in **pregnant rhesus monkeys** *in vivo*.

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N.8.1.2 Chicken blood pressure

PURPOSE AND RATIONALE

Oxytocin induces a transient fall in blood pressure in chicken and other birds. This effect can be used as assay for oxytocin (Coon 1939). The method has been modified by Munsick et al. (1960) and accepted by pharmacopoeias, e.g., the United States Pharmacopeia 23 (1995) uses the chicken blood pressure method for determination of oxytocin activity with three doses of standard and test preparation and calculation of the activity ratio with confidence limits.

PROCEDURE

White Leghorn chickens weighing 1.2 to 2.0 kg are anesthetized by intravenous injection with 200 mg/kg sodium phenobarbital via the brachial vein. The ischiadic artery is exposed by removing the feathers from the outer surface of the left thigh, an incision 7–8 cm long is made in the skin, parallel to and about 1.5 cm below the femur, exposing the gluteus primus muscle. The lower edge of this incision is retracted to expose the edge of the gluteus primus muscle overlying the semitendinosus muscle. The edge is then freed for the length of the incision, and when the free edge is lifted, the ischiadic artery, the ischiadic vein, and the crural vein can bee seen lying along the edge of semitendinosus muscle. The gluteus primus muscle is cut at right angles near the proximal end of the incision and the resulting flap deflected and secured to the upper thigh. Length of the ischiadic artery and crural vein are dissected free and the artery is cannulated. The cannula is connected to a Statham pressure transducer. Blood pressure should be between 100 and 120 mm Hg. The

crural vein is cannulated for injections of the test preparations. Intravenous injection of oxytocin induces in chickens an immediate, transient fall in blood pressure. Doses of the standard are chosen which are followed by a decrease of blood pressure between 20 and 40 mm Hg. The required doses normally lie between 20 and 100 mU.

Two doses of the standard and two doses of the test preparations are injected according to a randomized block or to a Latin square design and at least six to eight responses to each should be recorded. The interval between injections should be constant and lie between 3 and 10 min, depending on the rate at which the blood pressure returns to normal.

EVALUATION

The responses of each dose are averaged and potency ratios with confidence limits calculated from the 2 + 2-points assay.

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N.8.1.3 Milk ejection in the lactating rabbit or rat

PURPOSE AND RATIONALE

A sensitive bioassay for the estimation of oxytocin was described by van Dyke et al. (1955). This test makes use of the milk-ejecting properties of oxytocin.

PROCEDURE

A rabbit in the first or second week of lactation is anesthetized with urethane and pentobarbital. Usually, artificial respiration is not necessary. One jugular vein is cannulated for injections. One of the six ducts in a nipple of the rabbit is cannulated with a hypodermic needle and connected with a Statham strain gauge transducer. Two doses of the standard and the test preparation are injected according to a randomized block or to a Latin square design and at least six to eight responses to each should be recorded. The interval between injections should be constant at 3 min.

EVALUATION

The responses of each dose are averaged and potency ratios with confidence limits calculated from the 2 + 2-points assay.

MODIFICATIONS OF THE METHOD

The British Pharmacopoeia (1988) recommends the measurement of milk-ejection pressure in the lactating rat. A lactating rat weighing about 300 g in the third to twenty-first day after parturition is anesthetized by pentobarbitone sodium. The trachea is cannulated. One jugular or femoral vein is cannulated for injections of the test preparations. The tip of one lower inguinal teat is excised and a polyethylene tube with an external diameter of 0.6 mm is inserted to a depth sufficient to obtain appropriate measurement of pressure into the primary teat duct which opens onto the cut surface and tied firmly in place with a ligature. The cannula is connected with a suitable strain-gauge pressure transducer for recording on a polygraph.

Tindal and Yokoyama (1962) recommended the use of guinea pigs using essentially a similar procedure, but the injection is made into the internal saphenous artery after ligation of the main branches supplying the limb. In the guinea pig the mammary glands are supplied with blood from the external pudendal arteries which branch from the internal saphenous artery just as they enter the legs.

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N.8.1.4 Oxytocin receptor determination

PURPOSE AND RATIONALE

Premature labour and pre-term delivery is an important cause of death among infants and a major cause of newborn and child morbidity. Strong evidence exists that oxytocin and catecholamines are involved in the spontaneous uterine contractions which bring about premature delivery. Development of oxytocin antagonists is believed to be of therapeutic value for the prevention of pre-term labor (Manning et al. 1995; Chan et al. 1996).

In vitro binding to the oxytocin receptor is used as the first step of characterization of potential oxytocin antagonists (Pettibone et al. 1990, 1991, 1993a,b, 1996; Evans et al. 1993; Manning et al. 1995; Freidinger and Pettibone 1997; Pettibone and Freidinger 1997).

PROCEDURE

Uterine tissue is taken from nonpregnant adult Sprague Dawley rats pretreated (18–24 h) with diethylstilbestrol (300 µg/kg, i.p.) and mammary tissue from lactating rats (4–14 days lactation). The tissues are homogenized in 10 mM Tris containing 1 mM EDTA and 0.5 mM dithiothreitol, pH 7.4 and centrifuged at 48 000 *g* for 30 min at 4 °C. The resulting pellets from mammary/uterine tissue are resuspended in 50 mM Tris/5 mM $MgCl_2$/0.1% BSA (pH 7.4) and centrifuged again to produce the final pellet. Competition studies are conducted at equilibrium for 60 min at 22 °C using 1 nM [^{3}H]OT (30–60 Ci/mmol, New England Nuclear, Boston, USA) in the following buffer: 50 mM Tris/5 mM $MgCl_2$/0.1% BSA (pH 7.4). Nonspecific binding (5–10% of total binding) is determined using 1 µM unlabeled oxytocin. IC_{50} values are calculated from the linear regression analysis of log concentration of inhibitor versus percent inhibition of specific binding. Saturation binding studies are conducted at equilibrium using a 100-fold range of radioligand concentrations (i.e., 0.1 nM to 10 nM) and analyzed by a non-linear regression program (McPherson 1985a,b). The binding reactions are initiated by the addition of the tissue preparation (final protein concentrations, 100–200 µg protein/ml) and terminated by rapid filtration through Skatron glass fiber filters using a Skatron cell harvester system (Model 7019, Skatron Inc., Sterling, USA).

EVALUATION

Inhibition constants (K_i) are calculated for each compound from 3–6 separate IC_{50} determinations ($K_i = IC_{50} / [1 + c / K_d]$) using mean dissociation constants (K_d) obtained from saturation binding assays.

MODIFICATIONS OF THE METHOD

Maggi et al. (1994) used cultured Hs 805.Ut (corpus uteri, normal, human) cells or cells obtained from women in the early follicular or late luteal phase to study binding of antagonists at the human oxytocin receptor.

Pak et al. (1994) compared the binding affinity of oxytocin antagonists to human and rat oxytocin receptors and correlated the results with the rat oxytocic bioassay.

Species differences in central oxytocin receptor gene expression were investigated by Young et al. (1996).

Cloning and expression of the rhesus monkey oxytocin receptor was reported by Salvatore et al. (1998).

Molecular cloning and functional characterization of the oxytocin receptor from a rat pancreatic cell line (RINm5F) was reported by Jeng et al. (1996).

Elands et al. (1987), Klein et al. (1995) recommended selective radioligands for the oxytocin receptor.

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N.8.2
Vasopressin

N.8.2.1
Hereditary vasopressin deficiency in rats (Brattleboro strain)

This bioassay is based on an animal strain with genetic deficiency of vasopressin synthesis. Patients with diabetes insipidus excrete large amounts of very diluted urine and need a high fluid intake. This disorder is due to a lack of vasopressin. A similar syndrome has been found in Brattleboro rats. In the rare nephrogenic form, the vasopressin receptors are defective. Vasopressin is also considered to be a modulator in the central nervous system in particular learning and memory processes. Therefore, many studies on learning and memory have been performed with this strain of rats. Valtin et al. (1965) found very little vasopressin in hypothalami and pituitaries of homozygous Brattleboro rats.

Schmale and Richter (1984), Schmale et al. (1984) found a single base deletion in the vasopressin gene as the cause of diabetes insipidus in Brattleboro rats. The mutant vasopressin gene is transcribed but the message is not efficiently translated. Spontaneous hypertensive rats crossbred with Brattleboro rats inherit the mutated vasopressin gene (McCabe et al. 1988). The abnormal quinine drinking aversion in the Brattleboro rat with diabetes insipidus can be reversed by an vasopressin agonist (Laycock et al. 1994)

For testing aquaretic effects in Brattleboro rats see C.1.2.1.

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N.8.2.2 Vasopressor activity

PURPOSE AND RATIONALE

Vasopressin activity can be determined by the increase of blood pressure after surgical or pharmacological elimination of the central and peripheral nervous regulation of the cardiovascular system. Dekanski (1952) described a quantitative assay of vasopressin in anesthetized rats after blockade of other pressor substances by dibenamine (pithed rat).

PROCEDURE

A male Wistar rat weighing about 300 g is anesthetized by subcutaneous injection of 1.75 g/kg urethane. After 45–60 min, the trachea is cannulated with a polyethylene tube of 2.5 mm diameter. One femoral vein is cannulated for injections and the carotid artery for measuring blood pressure with a Statham transducer. The central and peripheral nervous systems, including both vagi and associated sympathetics, are left intact. No artificial respiration is necessary. Heparin (2 000 U/kg) is injected through the venous cannula and washed through with saline. Dibenamine = *N*-(2-chloroethyl)dibenzylamine hydrochloride is injected twice intravenously at an interval of 10 min in a dose of 1 mg/kg. The blood pressure stabilizes at a basal level of about 50 mm Hg. Small doses of vasopressin induce an increase in blood pressure which is dependent on the dose. Two doses of vasopressin standard (approximately 3–5 milliunits) and two doses of test preparation are injected repeatedly (usually 6 times) using a Latin square design. The doses are injected at intervals of 10–15 min.

EVALUATION

The responses of each dose are averaged and potency ratios calculated from the 2 + 2-points assay.

MODIFICATIONS OF THE METHOD

One of the first recommendations to standardize posterior pituitary extract was measuring blood pressure in anesthetized dogs (Hamilton 1912).

Vogel and Hergott (1963) studying the properties of a synthetic vasopressin analogue described a method in decerebrated rabbits previously used in this laboratory for standardization of posterior pituitary extracts. Rabbits weighing 2–3 kg are anesthetized by slow intravenous injection of butallylonal. The trachea is cannulated and the cannula connected with a respiration pump. One femoral vein is cannulated for injection of the test compounds. One carotid artery is cannulated for measurement of blood pressure with a Statham transducer. For chemical decapitation, the head of the anesthetized rabbit is bent forwards and a needle introduced into the foramen occipitale magnum. 0.5 ml of 30% trichloroacetic acid are injected. Artificial respiration is started immediately. Blood pressure is stabilized at a level of 30–40 mm Hg. Two doses of standard and of test preparation are injected according to a Latin square design. The method has been proven to be very sensitive.

The British Pharmacopoeia 1988 recommends the intravenous injection of 10 mg/kg of the α-adrenoreceptor blocking agent phenoxybenzamine hydrochloride 18 h prior to the experiment to stabilize the blood pressure on a low level. USP 23 (1995) uses two doses of standard and test preparation in the phenoxybenzamine blocked rat for calculation of vasopressin activity with confidence intervals in vasopressin and oxytocin preparations.

Knape and van Zwieten (1988) used the pithed rat to study vasoconstrictor activity of vasopressin after pretreatment with various drugs.

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N.8.2.3 Antidiuretic activity in the conscious rat

PURPOSE AND RATIONALE

For some period of time, it was uncertain whether or not vasopressin and adiuretin are the same or separate hormones. Special tests for antidiuretic activity in water loaded rats were developed (Burn 1931) and adopted by pharmacopoeias. Vasopressin analogues show considerable dissociation of vasopressive and antidiuretic activity (Vogel and Hergott 1963).

PROCEDURE

Wistar or Sprague-Dawley rats weighing between 140 and 250 g of either sex are used. The range of weights in any one test should not exceed 50 g. Not less than 2 days before testing the preparation, the rats have to be accustomed to the metabolism cage by carrying out a preliminary test. All animals are injected with 0.1 ml saline solution per 100 g body weight instead of the test preparations given in the main test. Any rat that shows signs of stress or undue excitement or that has abnormally low or high rate of urine excretion should not be used in the main test. Food and water is withheld during each test but access allowed between the tests. The rats are assigned at random to four groups each of them not less than four animals, weighed and marked for identification purposes. By stomach tube each rat receives a volume of water warmed to approximately 37 °C and equivalent to 5% of the animal's body weight. Each rat is placed in a separate cage for collection of urine. Thirty min later, the volume excreted by each rat is recorded and a second volume of tap water equal to the volume of urine together with a further volume equivalent to 3% of the animal's body weight is administered. This provides a total water load equivalent to 8% of the animal's body weight.

Using a different group of rats two dilutions of the preparation to be examined and of the standard preparation, immediately after administration of the second dose of water, each rat is injected subcutaneously with a volume of the appropriate dilution equivalent to 0.1 ml per 100 g body weight. The urine passed during the first 5 min after injection is discarded and the volumes collected at intervals of 15 min are noted until a volume greater than 30% of the total water load is excreted.

EVALUATION

The responses of each dose are averaged and activity ratios with confidence limits calculated from the 2 + 2-points assay.

MODIFICATIONS OF THE METHOD

Hydrated **conscious dogs** have been used to test the antidiuretic activity of vasopressin by van Dyke et al. (1955).

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N.8.2.4 Antidiuretic activity in the rat in ethanol anesthesia

PURPOSE AND RATIONALE

This is a sensitive bioassay often reported for vasopressin analogues. Ethanol suppresses the excretion of endogenous antidiuretic hormone (van Dyke and Ames 1951). Sensitive methods measuring urine output in ethanol anesthesia were described (Dicker 1953; Dettelbach 1958; Berde and Cerletti 1961), whereby the water load is automatically kept constant.

PROCEDURE

Female Sprague-Dawley rats weighing 200–250 g are spayed 7 to 10 days prior to use in order to eliminate the effect of gonadal steroids on the response to a water load. Each animal is given a 5 ml gavage of tepid tap water. At 3-days intervals thereafter, the water load is increased by 1 ml increments until a 10-ml level is reached. The day before an assay is to be performed, two or three animals are selected and given a 10 ml gavage of 2% ethanol. The animals are fasted overnight but are allowed free access to water. On the test day, they are placed into individual metabolism cages for water loading and anesthetizing. Each rat receives 5% of its body weight of warm 12–15% ethanol by stomach tube. This is anesthetic within a few minutes. After 45 min the same volume of 2% ethanol is again given by gavage. After 1 h the bladder is emptied by suprapubic pressure and the total urine output is measured.

The animal with the largest volume is selected and the water load calculated as the total volume administered less the volume excreted. The load on this animal is now

increased to between 6 and 8% of its body weight with warm 2% ethanol. Then the urinary bladder is catheterized. A polyethylene tubing is inserted into the rat's stomach. A femoral vein is cannulated for injections. The hind legs are secured to the operation table, while the front legs are left free. Assays are started when urine flow reaches a steady level of at least 50 µl/min. The catheter from the bladder is connected to an apparatus consisting of two Woulff bottles. Urine is directed through the catheter into the first Woulff bottle, displacing fluid into the second Woulff bottle. The second bottle is filled with 0.5% NaCl solution in 50% ethanol to reduce the size of the drops. Each drop activates an impulse counter. The drops are collected in a small glass reservoir and led by means of a polyethylene tubing to the urine metering pipette. When the fluid level in this tube rises and makes contact with an adjustable needle electrode near the top, another pipette which is calibrated to the same volume empties a solution of 5% glucose in 2% ethanol through the gavage tube into the rat's stomach. By these means a constant water load is maintained.

The animals produce urine at a relatively steady rate for 3–5 h. Intravenous injections of vasopressin produce characteristic changes in rate of urine flow which begin usually within 2 min and subside in most instances within 15 min. The range in which the assay of antidiuretic activities is useful is between 2 and 64 µU/injection.

EVALUATION

Antidiuretic potency is calculated as the log ratio of the volume excreted in 5 min preceding injection to the volume excreted in the 5 min beginning 1 min after injection. Dose-responses are established for the standard and the test preparation and activity ratios calculated.

MODIFICATIONS OF THE METHOD

Berde and Cerletti (1961) placed the rat anesthetized with ethanol on a balance. The water loss due to urine excretion and perspiratio insensibilis is replaced automatically using a solution of 5% glucose in 2% ethanol.

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N.8.2.5 Spasmogenic activity of vasopressin in the isolated guinea pig ileum

PURPOSE AND RATIONALE

The ileum of the guinea pig has been found to be quite sensitive to vasopressin (Simon 1933). This is a direct effect on smooth muscle similar to the pressor effect. This test is not specific for vasopressin.

PROCEDURE

Pieces of guinea pig ileum are suspended in an organ bath according to the Magnus' technique (see J.4.3.1). Two different doses of the test preparation and of the standard are applied according to a Latin square design and the contractions measured using a strain gauge transducer. Four–six doses of each solution are measured.

EVALUATION

The responses of each dose are averaged and activity ratios with confidence limits calculated from the 2 + 2-points assay.

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N.8.2.6 Vasopressin receptor determination

PURPOSE AND RATIONALE

As in other areas of drug research, identification of new vaopressin-like compounds has been achieved not by bioassays, but using receptor preparations. Such assays are also used in "receptor screens" for the general pharmacology of new drug candidates, to predict their spectrum of activities.

Arginine vasopressin exerts its action through three membrane bound G-protein-coupled receptor subtypes (V_{1a}, renal V_2 and V_3). The vasopressin-induced antidiuresis (via V_2 receptors coupled to aquaporins) helps maintain plasma osmolality and salt (NaCl) balances. The human V_1, V_2 and V_3 (V_{1b}) receptors and water selective membrane proteins (aquaporins) in the kidney have been cloned.

Pharmacological characterization of the human vasopressin receptor subtypes stably expressed in Chines hamster ovary cells was reported by Tahara et al. (1998).

Selective non-peptide vasopressin antagonists (without agonistic activity) have been shown to be aquaretic agents in animals and humans. Development and therapeutic indications of orally-active vasopressin receptor antagonists were reviewed by Thibonnier (1998).

Serradeil-Le Gal et al. (1993, 1994a) studied the biochemical and pharmacological properties of a non-peptide antagonist on rat and human vasopressin V_{1a} receptors.

PROCEDURE

Tissue samples from human uterus, adrenals, kidneys and pituitaries are collected in conformity with national ethical rules. Uterus, adrenal and kidney samples are immediately chilled in cold saline. Membranes are prepared within 3 h after collection. Pituitaries are collected within 6 h after death and immediately frozen in liquid nitrogen. Bovine kidneys can be obtained from a local slaughterhouse. Rat mammary tissue is taken from 19-day Sprague Dawley pregnant rats.

For human adrenal membrane preparations, pieces of adrenal glomerulosa zone are suspended in a cold buffer (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 1 mM PMSF) and fragmented using a glass/glass Dounce homogenizer. The homogenate is filtered through glass wool and centrifuged for 15 min at 1 500 *g* at 4 °C. The pellet is incubated for 15 min in ice-cold hypotonic buffer (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA, and 1 mM PMSF) and kept on ice to allow cell lysis. Lysed cells are recovered by centrifugation at 1 500 *g* for 15 min at 4 °C, homogenized using a loose-fitting Dounce homogenizer in hypotonic buffer supplemented with 40% glycerol, and stored at –20 °C. Before experiments, glycerol is eliminated by washing the membranes in glycerol-free hypotonic buffer.

For preparation of hypophyseal membranes, frozen entire pituitary glands are rapidly thawed at 37 °C in isotonic buffer supplemented with 0.1 mM PMSF, and the adenohypophyses are separated. Adenohypophyseal membranes are prepared as described above for adrenal membranes.

For rat mammary gland membrane preparations, tissues are minced and homogenized in 50 mM 10% (wt/vol) buffer A Tris-HCl, pH 7.4, 320 mM sucrose, and 0.5 mM dithiothreitol and centrifuged at 900 *g* for 15 min; the pellet is resuspended in buffer A and centrifuged as above. The two 900-g supernatants are filtered through cheesecloth and centrifuged at 70 000 *g* for 20 min. The pellet is washed with buffer B containing 50 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$. Finally, the 70 000 *g* washed pellet is suspended in buffer B at a final concentration of ~8 mg protein/ml and stored in aliquots in liquid nitrogen.

V_{1a} binding assays using ^{125}I linear AVP antagonist on human adrenal membranes, myometrial membranes from nonpregnant uterus, or platelet membranes are performed in an incubation medium (200 µl) containing 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mg/ml BSA, 0.05 mg/ml soy bean trypsin inhibitor, 0.5 mg/ml bacitracin, 0.1 mM PMSF, 0.5 mM EDTA, 10–60 pM ^{125}I-AVP linear antagonist, and increasing amounts of test compound. The reaction is started by addition of membranes (10–40 µg/assay) that are incubated at 30 °C for 45 min. The reaction is stopped by adding 3 ml of ice-cold filtration buffer (10 mM Tris HCl, pH 7.4, and 3 mM $MgCl_2$ followed by filtration through GF/C Whatman glass microfiber filters that have been soaked for at least 5 h in a solution containing 10 mg/ml BSA. Filters are washed five times with 3 ml of filtration buffer and counted for radioactivity by gamma spectroscopy. Nonspecific binding is determined in the presence of 0.3 µM unlabeled iodinated AVP linear antagonist or 1 µM AVP.

EVALUATION

The IC_{50} value is defined as the concentration of inhibitor required to obtain 50% inhibition of the specific binding. Inhibition constant (K_i) values are calculated from the IC_{50} values using the Cheng and Prusoff equation. Data for equilibrium binding (K_d, B_{max}), competition experiments (IC_{50} nHill), and kinetic constants (k_{obs}, k_1) are analyzed using an iterative nonlinear regression program (Munson and Rodbard 1980).

MODIFICATIONS OF THE METHOD

A receptor assay for arginine-vasopressin has been described by Gopalakrishnan et al. (1986).

Pávó et al. (1993) reported synthesis and binding characteristics of two sulfhydryl-reactive probes for vasopressin receptors.

Serradeil-Le Gal et al. (1994b) tested the effect of a non-peptide vasopressin V_{1a} vasopressin antagonist on the binding and the mitogenic activity of vasopressin on Swiss 3T3 cells.

Yatsu et al. (1997) tested the vasopressin antagonistic activities of a non-peptide on V_{1a} receptors in dog platelets and on V_2 receptors in dog kidney homogenates.

Ogawa et al. (1996), Tahara et al. (1997a) tested V_{1a} receptor binding in homogenates of rat liver and V_2 receptor binding in rat kidney.

Barberis et al. (1995) characterized a linear radioiodinated vasopressin antagonist as an excellent radioligand for vasopressin V_{1a} receptors.

Radioligands for vasopressin V_1 receptors were described by Elands et al. (1988) and by Kelly et al. (1989).

Ala et al. (1997) reported the properties of a radioiodinated antagonist for human vasopressin V_2 and V_{1a} receptors and recommended this ligand for further studies on human vasopressin V_2 receptor localization and characterization, when used with a selective vasopressin V_{1a} ligand.

Howl and Wheatley (1995) found a species heterogeneity in the characteristics of V_{1a} receptors and in the expression of hepatic V_{1a} receptors.

Carnazzi et al. (1997) described photoaffinity labeling of the rat V_{1a} vasopressin receptor using a linear azidopeptidic antagonist.

Phalipou et al. (1997) studied the peptide-binding domains of the human V_{1a} vasopressin receptor with a photoactivatable linear peptide antagonist.

DDAVP (1-desamino-8-d-arginine vasopressin) which is considered as a standard V_2 vasopressin receptor selective agonist was found to act also as agonist on the V_{1b} vasopressin receptor (Saito et al. 1997).

Tahara et al. (1997b)investigated the effects of a nonpeptide V_{1a} and V_2 vasopressin receptor antagonist in binding and functional studies on rat vascular smooth muscle cells.

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N.9
Hypothalamic hormones

N.9.1
Thyrotropin releasing hormone (TRH)

N.9.1.1
General considerations

Hypothalamic regulatory hormones are used in diagnostic procedures and for therapy, usually modified by chemical synthesis to enhance activities. The existence of regulators of anterior pituitary function, already postulated many years ago (Bargmann 1949; Scharrer and Scharrer 1954), has been experimentally demonstrated for the first time by Saffran and Schally (1955) in experiments utilizing hypothalamic and neurohypophyseal extracts. TRH was the first hypothalamic hormone whose chemical structure was elucidated (Bøler et al. 1969; Schally et al. 1970). Its main use is as a diagnostic in thyroid disorders, pituitary tumors and infertility.

Cloning, characterization, and transcriptional regulation of the thyrotropin-releasing hormone gene was reviewed by Wilber and Ai-Hua-Xu (1998).

Pekary (1998) discussed the physiological role of thyrotropin-releasing hormone-enhancing peptide (Ps4) which results from proteolytic processing of prepro-TRH.

Effects of TRH and its analogues in the CNS not related to the release of TSH (extrapituitary effects) have been found indicating perhaps other therapeutic indications (Metcalf 1983; Flohe et al. 1983; Nemeroff et al. 1984; Horita et al. 1986; Horita 1998).

Radioimmunoassays for TRH are available (Bassiri et al. 1978).

Furukawa et al. (1980) reported the local effects of TRH on the isolated small intestine and taenia coli of the guinea pig. This effect could be related to the local release of catecholamines.

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N.9.1.2
TRH receptor binding assays

PURPOSE AND RATIONALE

Thyrotropin hormone releasing hormone receptors from mouse (Straub et al. 1990; Jones et al. 1996), from rat (Sellar et al. 1993) and from men (Duthie et al. 1993; Matre et al. 1993; Yamada et al. 1993; Hinuma et al. 1994) have been expressed and characterized. Two isoforms have been identified (de la Peña et al. 1992; Lee et al. 1995). Constitutive activity of native thyrotropin-releasing hormone receptors has been shown by Jinsi-Parimoo and Gershengorn (1997). Molecular and cellular biology of thyrotropin-releasing hormone receptors has been reviewed by Gershengorn and Osman (1996).

TRH receptors have been localized not only in the pituitary but also in other brain regions (Burt and Taylor (1983). TRH receptors can be determined by binding of [^{3}H]MeTRH (Taylor and Burt 1981; Sharif and Burt 1983; Jarowska-Feil et al. 1995; Yamada et al. 1995) or [^{3}H](3-Me-His^2)TRH (Simasko and Horita 1982).

PROCEDURE

Pooled tissue samples are homogenized in a sodium phosphate buffer (20 mM, pH 7.4) and centrifuged at 30 000 *g* for 30 min. The resultant pellets are washed twice by means of resuspension and centrifugation. The washed membranes are dispersed in fresh buffer and are used for the TRH receptor binding assay.

The membranes are incubated in 250 µl of the total volume with 0.5–8 nM of [^{3}H]MeTRH (NEN, sp. act. 62.8 Ci/mmol), in the presence or absence of 10 µM of TRH for 5–6 h at 0 °C (in a water-ice bath). The receptor bound and free [^{3}H]MeTRH is separated by a rapid filtration through a glass fiber filter GF/B (Whatman) under reduced pressure (Harvester-Brandel). The trapped receptor bound radioactivity is determined by liquid scintillation spectrometry (Beckman). The amount of specifically bound [^{3}H]MeTRH is expressed as fmoles per mg of protein.

EVALUATION

The data are subjected to the 6-point Scatchard analysis. The receptor density (B_{max}) and apparent dissociation constant (K_d) are determined.

MODIFICATIONS OF THE METHOD

Sun et al. (1998) described cloning and characterization of the chicken thyrotropin-releasing hormone receptor.

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N.9.1.3 Release of ^{131}I from thyroid glands of mice

PURPOSE AND RATIONALE

An animal bioassay method for TRH utilizes iodine-deficient mice, treated with ^{131}I, codeine, and 1 µg thyroxin (Redding et al. 1966; Bowers et al. 1967; Redding and Schally 1969). This is a historical test based on the effect of thyroid hormone release by TRH (indirect

bioassay). Provided the extract being tested is free of TSH, the rise in radioactivity in blood 2 h after injection of the TRH preparations is proportional to the effect of TSH released by TRH.

PROCEDURE

Weanling mice of the Swiss-Webster strain weighing 15 g are fed a low iodine diet and given distilled water for 10 days. At zero time, 4 μC $Na^{131}I$, 1 μg thyroxin and 1 mg codeine phosphate are injected subcutaneously. Codeine is given again s.c. at 24, 30, and 48 h after 0 time. Two h after the fourth injection of codeine, blood is taken from the orbital venous sinus. The test preparation or TRH standard is injected intravenously at increasing doses (0.01, 0.03, 0.09 μg/mouse). Two hours later, a second blood sample is taken from the orbital venous sinus. The response is obtained by the increase of radioactivity from the first to the second blood sample (Δcpm).

EVALUATION

Dose-response curves are established for test preparation and standard for calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

TRH also stimulates release of TSH and subsequently of ^{131}I in rats pretreated with $Na^{131}I$ and 5 μg thyroxin (Yamakazi et al. 1963).

TRH increases plasma TSH levels in thyroidectomized rats pretreated with 1 μg of T_3 (Bowers et al. 1965). This assay is based on enhanced TSH secretion after eliminating then feedback of thyroid hormones. The baseline is then lowered by T3 pretreatment to provide a wider range for stimulation by TSH. Rats weighing 350–400 g are surgically thyroidectomized. One to 3 months later, 1 μg of L-triiodothyronine Na is given i.p.; 2 h later urethane is given s.c.; approximately 1 h later 1.5 ml blood is removed from the jugular vein and the TRH preparation is administered i.v. Fifteen min later, another 1.5 ml blood is removed. Heparin is added to the blood and TSH levels in plasma are assayed by release of ^{131}I from the mouse thyroid (Mc Kenzie 1958) (see N.7.5.3). Results are recorded as mean change in blood ^{131}I levels of 5 mice.

TRH produces a significant depletion of pituitary TSH content in mice (Bowers et al. 1967). Swiss-Webster strain mice are treated as described for determination of blood ^{131}I radioactivity. Two hours after administration of TRF, the animals are decapitated and the pituitaries immediately removed. Pituitaries of each group of 5 mice are combined and homogenized in 5 ml of 0.01 M acetic acid which contains 0.9% NaCl. TSH is determined according to McKenzie (1958) (see N.7.5.3). Today, this would be replaced by RIA determination of pituitary TSH content.

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N.9.1.4 Release of TSH from rat anterior pituitary glands *in vitro*

PURPOSE AND RATIONALE

The *in vitro* bioassay method of Saffran and Schally (1955a,b), developed for detecting CRF activity, has been modified to measure TRH activity *in vitro* (Guillemin et al. 1963; Bowers et al. 1965; Schally and Redding 1967).

PROCEDURE

Male Sprague-Dawley rats weighing 150–200 g serve as donors. After removal each pituitary is cut in half, transferred to a 15 ml beaker containing 1.5 ml Krebs-Ringer bicarbonate medium with 200 mg% glucose

and incubated for three 60-min periods. The media used in the first 2 incubations are discarded. At the beginning of the third incubation period, various amounts of test preparation or TRH standard are added to individual beakers. At the end of the third incubation period the media from both control and experimental beakers are carefully freed of pituitary tissue. The media are then assayed by RIA for content of TSH.

EVALUATION

Dose-response curves are established for test preparation and standard allowing calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

For the assay of TRH analogues, cultures of enzymatically dispersed anterior pituitary cells from rats can be used instead of pituitary halves (Vale et al. 1972).

Barros et al. (1986) studied the effect of TRH on cultured GH3 rat anterior pituitary cells using the whole-cell voltage clamp technique.

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N.9.2
Luteinizing hormone releasing hormone (LH-RH)

N.9.2.1
General considerations

Gonadotropin secretion is controlled by the hypothalamic peptide LHRH, which stimulates the release of FSH and LH. The discovery of the structure of luteinizing hormone releasing hormone (LH-RH) then led to the synonym of gonadotropin releasing hormone (Gn-RH) and identification of its receptors followed (Conn et al. 1986). Nevertheless, the search for a specific FSH-releasing factor has continued (McCann et al. 1993). LH-RH is secreted in a pulsatile fashion (Levine et al. 1991). This however not of relevance for the bioassays which use injections as well as infusion for several hours. Radiolabeling and photoaffinity labeling of gonadotropin releasing-hormone receptors have been described (Perrin et al. 1982; Hazum and Keinan 1983). LH-RH bioactivity is determined *in vitro* on pituitary cells and *in vivo* by its effects on ovulation, spermatogenesis and other gonadal parameters (McCann 1970; Steelman 1970). Radioimmunoassays and radioreceptorassays are available (Nett and Niswender 1979). Many studies in rats and primates showed that prolonged administration of LH-RH agonists results in a decrease of LH and gonadal hormones to castrate levels (Sandow et al. 1978, 1980; Akhtar et al. 1983; Weinbauer et al. 1987, 1990).

Short- and long-term effects on pituitary-gonadal function in neonatal and adult female rats treated with gonadotropin-releasing hormones were analyzed by Trimino et al. (1993).

FDA recommendations for preclinical testing of gonadotropin releasing hormone (GnRH) analogues including pharmacology, pharmacokinetics and toxicology were published by Raheja and Jordan (1994).

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N.9.2.2 LH-RH receptor assays

PURPOSE AND RATIONALE

LH-RH (GnRH) interacts with a membrane receptor which belongs to the G-protein-coupled receptor family. The LH-RH-R is encoded by a single-copy gene consisting of three exons and two introns. Consisting with the site of its action, LH-RH-R mRNA has been found in the brain, pituitary, gonads, placenta, as well as in tumor tissue and tumor cell lines (Jennes and Conn 1994). Binding to LH-RH receptors in rat pituitary membranes has been studied for LR-RH agonists and antagonists. Furthermore, the time course of down-regulation of LH-RH receptors was followed (Halmos et al. 1996).

PROCEDURE

Receptor binding of LH-RH is determined using a sensitive *in vitro* ligand competition assay based on binding of radiolabeled buserelin or [D-Trp^6]LH-RH to rat anterior pituitary membrane homogenates (Halmos et al. 1993; Szöke et al. 1994; Halmos et al. 1996). Membrane homogenates containing 40–80 µg of protein are incubated in triplicate with 60 000–75 000 cpm (≈0.15 nM) [^{125}I] [D-Trp^6]LH-RH as radioligand and with increasing concentrations (10^{-12}–10^{-6} M) of non-radioactive peptides in a total volume of 150 µl of binding buffer. At the end of the incubations, 125-µl aliquots of suspension are transferred onto the top of 1 ml of ice-cold binding buffer containing 1.5% bovine serum albumin in siliconized polypropylene microcentrifuge tubes (Sigma). The tubes are centrifuged at 12 000 *g* for 3 min at 4 °C. Supernatants are aspirated, and the bottoms of the tubes containing the pellet are cut off and assayed in a γ-counter. Protein concentration is determined by the method of Bradford (1976) using a BioRad protein assay kit.

EVALUATION

Specific ligand binding capacities and affinities are calculated by the computerized curve-fitting program of Munson and Rodbard (1980) as modified by McPherson (1985). To determine the types of receptor binding, dissociation constants (K_d values), and the maximal binding capacity (B_{max}), LH-RH binding data are also analyzed by the Scatchard method. Statistical significance is assessed by Duncan's new multiple range test.

MODIFICATIONS OF THE METHOD

Flanagan et al. (1998) recommended ^{125}I-[His^5,Tyr^6]-GnRH as radioligand for analysis of mutant GnRH receptors.

Perrin et al. (1982) compared binding of radiolabeled antagonists and antagonists of gonadotropin-releasing hormone to rats anterior pituitary membrane homogenates.

Fekete et al. (1989) reviewed the role of receptors for luteinizing hormone-releasing hormone, somatostatin, prolactin, and epidermal growth factor in rat and human prostate cancers and in benign prostate hyperplasia.

Cloning, sequencing, and expression of human gonadotropin releasing hormone receptor was published by Kakar et al. (1992).

Marheineke et al. (1998) characterized the human gonadotropin-releasing hormone receptor heterologously produced using the baculovirus/insect cell and the semliki forest virus systems.

The binding kinetics of a long-acting gonadotropin-releasing hormone antagonist to rat LH-RH receptors were studied by Li et al. (1994).

Lovejoy et al. (1995) determined the receptor binding of gonadotropin-releasing hormone analogues in bovine pituitary membrane preparations.

The cDNA encoding the receptor for LH-RH was isolated from a human pituitary cDNA library and heterologously expressed in the murine fibroblast cell line LTK^- by Beckers et al. (1995).

Tsutsumi et al. (1995) investigated the role of altered receptor biosynthesis in agonist-induced receptor down-regulation in αT_3-1 cells, a mouse gonadotrope cell line.

Beckers et al. (1997) characterized gonadotropin-releasing hormone analogs by a cellular luciferase reporter gene assay. The assay is based on a fusion of the c-fos immediate-early gene promotor to Photinus pyralis luciferase (LUC) as reporter gene, stably transfected in murine LTK^- cells expressing the human GnRH receptor. Transcription of endogenous c-fos and fos-Luc fusion gene are transiently induced quite similar by fetal calf serum or a superagonistic GnRH analog. The reporter gene was used to monitor agonist induced signaling via the human GnRH receptor. Whereas Luc activity was induced in a dose-dependent manner by GnRH or an agonistic analog, different antagonistic peptides completely inhibited this stimulation.

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N.9.2.3 LH release in the ovarectomized estrogen-progesterone-blocked rat

PURPOSE AND RATIONALE

Ramirez and McCann (1963) recommended the ovarectomized, estrogen progesterone-blocked rat (OEP-rat) as a highly sensitive test model for LH-releasing activity. The gonadotropin content of the pituitary is increased after ovarectomy, due to reduced feedback inhibition by absence of gonadal steroids. Basal secretion is then acutely lowered by estrogen/progesterone blockade, whereas the amplitude for stimulation of LH secretion is augmented. The increase in plasma LH levels of donor rats after injection of LH-RH previously determined by bioassay in recipient rats is now conveniently measured by RIA. Since natural and synthetic LH-RH releases both gonadotropins(Schally et al. 1971b), merely stimulation of LH is necessary for routine LH-RH assay.

PROCEDURE

Female Sprague-Dawley rats weighing 150–200 are ovarectomized. They are kept in a light and temperature controlled animal room at 24 °C and are fed commercial rat chow and tap water ad libitum. Tests are performed 1–3 months after ovarectomy. Three days prior to the assay, the rats receive 50 µg estradiolbenzoate and 25 mg progesterone in sesame oil by the transmuscular-subcutaneous route. For the assay, the rats are anesthetized by subcutaneous injection of 0.6 ml/100 *g* of 25% urethane solution. Various doses of the test compound or the standard are injected intravenously in the jugular vein. Ten min later, 4–6 ml blood are withdrawn by cardiac puncture from each donor allowing the separation of 2 ml plasma.

In the original method, bioassayable LH was measured by the ovarian ascorbic acid depletion (OAAD) method according to Parlow (1963) (see also N.7.1.2.3). Immature female Sprague Dawley rats are injected on day 1 at 3:00 P.M. with 50 IU PMSG in 0.2 ml saline subcutaneously. On day 3, the rats receive at 9:00 A.M. 25 IU HCG subcutaneously. On day 7, three different doses of the standard (e.g., NIH LH-S-1, National Institute of Health, Bethesda, ML) or the 2 ml of plasma from OEP-rats are injected intravenously. Eight animals are used per group. Three hours later, the animals are sacrificed, both ovaries prepared, weighed and homogenized for determination of ascorbic acid content. The LH-activity in OEP-plasma as measure by OAAD, is expressed in terms of NIH-LH-S16 standard.

Furthermore, LH activity in the plasma of OEP-rats is measured by radioimmunoassay. OEP rats are anesthetized with 25% urethane i.p. Blood is withdrawn by retroorbital puncture. Then various doses of the test preparation or the standard are injected intravenously. After 10 min blood is withdrawn by cardiac puncture. Radioimmunoassays of LH are carried out by the double-antibody-method of Niswender et al. (1968).

EVALUATION

Dose-response curves are established of the standard and the test preparation of LH-RH measured by bioassayable LH in OEP plasma as well as for plasma-RIA-LH level allowing the calculation of potency ratios with confidence limits. Furthermore, time-response curves can be established by determination of plasma-RIA-LH levels in OEP rats.

MODIFICATIONS OF THE METHOD

Instead of OEP rats, normal Male Sprague-Dawley rats can be used for measurement of the time course of release of LH after injection of LH-RH or LH-RH derivatives (Arimura et al. 1972). Male Sprague-Dawley rats weighing 120–150 g are anesthetized with 0.6 ml/100 g body weight of 25% urethane solution subcutaneously. After 30 min 0.8 ml blood is withdrawn from the jugular vein being immediately substituted by the same volume of Haemaccel® solution. The LH-RH preparation is injected subcutaneously in 1% gelatine-saline. Blood is withdrawn at hourly intervals up to six hours each time being substituted with Haemaccel® solution. LH in plasma is determined by the double-antibody-method of Niswender et al. (1968) and FSH according to the method of Daane and Parlow (1971).

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N.9.2.4 Gonadotropin release from anterior pituitary cells

PURPOSE AND RATIONALE

Anterior pituitaries can be used directly, kept in culture or used for cell lines (Mittler and Meites 1964, 1966; Mittler et al. 1970; Sandow et al. 1972) in order to study the synthesis and release of gonadotropins in response to LH-RH.

PROCEDURE

Female Sprague-Dawley rats weighing 100–150 g are used as donors. Each anterior pituitary is removed and cut into 4–6 pieces of approximately equal size. The cultures are performed in 3.5–1-cm sterile disposable plastic Petri dishes each containing 3 ml medium consisting of 9 parts Difco medium 199 and 1 part of newborn calf serum. Twenty-five U/ml penicillin and 25 µg/ml streptomycin are added. In each dish, the explants are supported at the gas interface. An atmosphere of 95% oxygen and 5% carbon dioxide and a temperature of 36 °C are maintained. Opposite sides of the same pituitaries provide matched control and experimental preparations. The pituitaries are incubated for a total time of 5 days. After the first two days, the medium is removed and discarded. Fresh medium is then added with the LH-RH solutions. Approximately 12 h after the first change of medium and addition of LH-RH, media are removed and frozen. Fresh medium with LH-RH is again added; this procedure is repeated until 6 samples of medium representing the last 3 days of culture are obtained. Media are assayed for LH content by radioimmunoassay according to Niswender et al. (1968) and for FSH content according to Parlow et al. (1963).

EVALUATION

Using various concentrations of test preparation and LH-RH standard dose-response curves are obtained allowing calculation of potency ratios with confidence limits.

CRITICAL ASSESSEMNT OF THE METHOD

These methods do not reflect the time course of release found *in vivo* but are useful for potency estimates.

MODIFICATIONS OF THE METHOD

Instead of pituitary halves for the assay of LH-RH as well as for the assay of TRF and its analogues, cultures of enzymatically dispersed anterior pituitary cells from rats can be used (Vale et al. 1972; Martin and Sattler 1979).

Loughlin et al. (1981) used perifused pituitary cultures as model for LH-RH regulation of LH secretion.

O'Connor and Lapp (1984) studied the effect of pulse frequency and duration of luteinizing hormone releasing hormone in anterior pituitary cells attached to Cytodex I beads.

Functional integrity of anterior pituitary cells separated by a density gradient has been studied (Scheikl-Lenz et al. 1985).

Receptor binding ability to rat pituitary and human breast cancer membranes of different agonists and antagonists of luteinizing hormone-releasing hormone was studied by Fekete et al. (1989).

Vigh and Schally (1984), Csernus and Schally (1991) described in detail a cell superfusion system consisting of a Sephadex column with dispersed pituitary cells. LH response of anterior pituitary cells to 3 min exposure to various concentrations of LHRH at 30 min intervals as well as growth hormone response to human growth hormone releasing hormone resulted in an excellent dose-response curves. The effect of growth hormone releasing hormone was inhibited by somatostatin. Likewise, the effect of LHRH was inhibited by pretreatment with LHRH antagonists.

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N.9.2.5 Radioimmunoassay of rat-LH

PURPOSE AND RATIONALE

Luteinizing hormone is a glycoprotein hormone exhibiting species specificity. For pharmacological experiments in rats, such as evaluation of gonadotropin releasing hormone activity, a homologous assay is necessary. The reagents are provided by the National Pituitary Agency, Bethesda, Md. The assay procedure is similar to the standard operating procedure proposed by the National Pituitary Agency, USA.

PROCEDURE

Reagents

Standard:	NIH-rat-LH-RP 1
Antiserum:	rabbit-anti-rat-S 9
Tracer:	^{125}I-rat-LH-I 6
Second antibody:	Behring goat-anti-rabbit-gamma-globulin, (Behring, Cat. No. OTOP 14/15)
Buffer:	0.01 M-phosphate-saline pH 7.4

Standard and samples are dissolved in 1% bovine serum albumin, tracer in 0.1% bovine serum albumin, and antiserum in EDTA-PBS (1 : 350 normal rabbit serum as carrier).

Assay

Standards:	0.25–62.5 ng/tube, 200 µl/tube
Antiserum:	1 : 20 000 100 µl/tube,
Tracer:	specific activity 120 µCi/µg, 8 000 cpm in 100 µl/tube

Standards (or sample) are incubated with antiserum and tracer for 48 h at +4 °C, the second antibody 1 : 50, 200 µl/tube is added, and incubated for 24 h at +4 °C. Separation is performed with 1.0 ml ice-cold phosphate buffered saline pH 7.4, the vial spun at 1 300 *g* for 15 min, the supernatant decanted, and the residue counted for 1 min in a gamma-counter.

EVALUATION

Data processing: standard curves and sample data are calculated on a computer program using a spline function.

Quality control parameters

Limit of detection:	0.36 ng per tube
Standard curve (ED_{80}-ED_{50}-ED_{20}):	1.31-4.56-15.74 ng per tube
Inter-assay CV (15 assays):	20.9%
Intra-assay CV:	<15 %

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N.9.2.6 Radioimmunoassay of rat-FSH

PURPOSE AND RATIONALE

Follicle-stimulating hormone is a glycoprotein hormone exhibiting species specificity. For pharmacological experiments in rats, such as evaluation of gonadotropin releasing hormone activity, a homologous assay is necessary. The reagents and the procedure are provided by the National Pituitary Agency, Bethesda, Md. The assay procedure is similar to the standard operating procedure proposed by the National Pituitary Agency, USA.

PROCEDURE

Reagents

Standard:	NIAMDD-rat-FSH-RP-1
Antiserum:	rabbit-anti-rat-FSH (NIAMDD-S-9)
Tracer:	^{125}I-rat-FSH (e.g. NIAMDD-I-4)
Second antibody:	Behring goat-anti-rabbit-gamma-globulin, (Behring, Cat. No. OTOP 14/15)
Buffer:	0.01 M-phosphate-saline/0.1% bovine serum albumin, pH 7.4

Assay

Standards:	6.25–1 600 ng/tube, 200 µl/tube
Antiserum:	1 : 2 000 100 µl/tube,
Tracer:	specific activity 200 µCi/µg, 10 000 cpm in 100 µl/tube

Standards (or sample) are incubated with antiserum and tracer for 72 h at +4 °C, the second antibody 1 : 50, 200 µl/tube is added, and incubated for 48 h at +4 °C. Separation is performed with 1.0 ml ice-cold phosphate buffered saline pH 7.4, the vial spun at 1 300 *g* for 15 min, the supernatant decanted, and the residue counted for 1 min in a gamma-counter.

EVALUATION

Data processing: standard curves and sample data are calculated on a computer program using a spline function.

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N.9.2.7 Measurement of ascorbic acid depletion in ovaries of pseudo-pregnant rats

PURPOSE AND RATIONALE

The assay for LH-RH and LH-RH analogues can be performed in one step in pseudopregnant immature female rats using the biological response of the luteinized ovaries to gonadotropins released by the test compounds. Pseudopregnancy is induced in immature female Sprague-Dawley rats by treatment with gonadotropins. Numerous corpora lutea are formed after ovulation. On day 7–8 after start of the treatment the corpora lutea are very sensitive to endogenous or exogenous gonadotropins. Stimulation of steroid synthesis, mainly progesterone, is seen associated with dose dependent ascorbic acid depletion in the ovaries. The activity of LH-RH and LH-RH analogues can be determined by the decrease of ovarian ascorbic acid concentration, or increase in progesterone secretion. Ascorbic acid depletion is a sensitive parameter for the endogenous gonadotropin release in the animals.

PROCEDURE

Immature female Sprague-Dawley rats weighing 35–45 g are pretreated with 50 IU PMSG followed by an injection of 25 IU HCG on the third day. On day 7 or 8, they are injected i.m. or s.c. with the test preparation or the LH-RH standard in 0.1 ml 1% gelatine-saline. Eight animals are used for each of three doses of test preparation and standard. One hour later, the ovaries are dissected out, homogenized, and ascorbic acid is determined according to the method of Mindlin and Butler (1938) by photometry.

EVALUATION

Dose-response curves of ascorbic acid depletion are obtained for test preparation and standard allowing the calculation of potency ratios with confidence limits.

MODIFICATION OF THE METHOD

The method has been adopted as biological assay of gonadorelin with some modifications by pharmacopoeias, e.g. the British Pharmacopoeia 1988.

REFERENCES

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Sandow J, v Rechenberg W, Jerzabek G (1976) The effect of LH-RH, prostaglandins and synthetic analogues of LH-RH on ovarian metabolism. Eur J Gynec Reprod Biol 6:185–190

N.9.2.8 Progesterone production in pseudopregnant rats

PURPOSE AND RATIONALE

Pseudopregnancy is induced in immature rats by pretreatment with gonadotropins. Numerous corpora lutea are formed after ovulation which are sensitive to endogenous gonadotropins released by LH-RH or LH-RH analogues on day 6–8 after treatment. By measurement of plasma progesterone, the steroidogenic activity of LH-RH analogues can be determined.

PROCEDURE

Immature female Sprague-Dawley rats weighing 35–45 g are pretreated with 50 IU PMSG (Pregnant mare serum gonadotropin) followed by an injection of 25 IU HCG (human chorionic gonadotropin) on the third day. They are injected intramuscularly with LH-RH or the LH-RH analogue on days 6,7, or 9 between 8 and 10 A.M. Eight animals are used for each of three doses of test preparation and standard. Blood samples are collected 1 h after treatment. Plasma samples of equal volume are extracted with peroxide free diethylether. The ether phase containing progesterone is evaporated and the sample re-dissolved in BSA-phosphate buffer. Tritium labeled progesterone and a specific antiserum against progesterone are added and incubated over a period of 24 h at 4 °C. Bound hormone and free hormone are separated by absorption on dextran coated charcoal. The activity of the sample is determined in a scintillation cocktail containing Triton X.

EVALUATION

Dose-response curves of progesterone concentrations are established for test preparation and standard allowing the calculation of potency ratios with confidence limits.

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N.9.2.9 Induction of ovulation in rabbits

PURPOSE AND RATIONALE

Ovulation can be induced in mature rabbits by injection of LH-RH after initial priming. This is a modified assay for LH release.

PROCEDURE

Six unmated, mature female rabbits (Himalayan strain) weighing 1,0 to 1,2 kg are used for each dose of standard or test preparation. Follicular maturation is induced by 8 subsequent daily subcutaneous injections of LH-RH or the LH-RH analogue to be tested in 0.2 ml 1% gelatine-saline. Forty-eight hours after the last injection, ovulation is induced by administering a four- to tenfold higher dose of the peptide in 0.2 ml subcutaneously. Twenty-four hours later, the animals are sacrificed, the ovaries weighed and ovulation is determined by counting the numbers of follicles ovulated in the ovaries, as defined by local bleeding.

EVALUATION

At least two doses of test preparation and standard are used in order to establish dose-response curves allowing calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Schröder et al. (1972), Sandow and Hahn (1973) described changes in the ovary transplanted to the anterior eye chamber in the rabbit enabling the direct observation of ovulation. This method is also applicable to steroids and prostaglandins.

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N.9.2.10 Induction of superovulation in immature rats

PURPOSE AND RATIONALE

Immature female rats (24–26 days of age) do not show vaginal cycles before the onset of puberty. Treatment with PMSG induces follicular maturation, followed by spontaneous ovulation 2 days later. Spontaneous ovulation can be blocked by barbiturates (e.g., phenobarbital) acting on the hypothalamus, and is overcome by exogenous LHRH. The rats ovulate due to the release of endogenous gonadotropins. After hypophysectomy, no ovulatory activity is observed in the absence of the pituitary.

PROCEDURE

Immature female Sprague-Dawley rats weighing 55–65 g are injected on day 1 with 10 IU PMSG subcutaneously. Following this pretreatment, the animals will ovulate spontaneously on day 3 between 2:00 and 4:00 P.M. Spontaneous ovulation is blocked by phenobarbital 4 mg/kg at 1:00 P.M. One hour later, the ovulatory peptide, dissolved in 1% gelatine/saline, is injected intravenously. Controls are treated with 1 or 2 IU HCG, negative controls with gelatine-saline only. Eight rats are used for control groups and various doses of LH-RH or the LH-RH analogue. On the next day, the rats are sacrificed at 11:00 A.M. and the oviducts are dissected and stained with Patent blue. The number of ova is counted under a microscope.

EVALUATION

A dose-dependent increase of ova per rat is observed after LHRH due to LH release and after HCG by direct action on the ovary. The effect of LH-RH analogues is compared with the effects of HCG. Minimal effective doses for LH release can be calculated.

REFERENCES

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N.9.2.11 Inhibition of experimentally induced endometriosis

PURPOSE AND RATIONALE

This is an example of a disease model in animals designed to mimic treatment of an experimentally induced pathological condition. Endometriosis-like lesions can be induced in female rats by autotransplantation of endometrium under the renal capsule (Sakata et al. 1990; Mizutani et al. 1995). This method was used to compare the effect of steroid-suppression by LHRH analogue after chronic administration (pituitary inhibition).

PROCEDURE

Under anesthesia laparatomy is performed in 9-week old female Sprague Dawley rats. The left uterine horn is resected and opened by a longitudinal incision. The endometrium is dissected from the myometrium. Then a 5×5 mm section of the endometrium is grafted under the capsule of the left kidney of the same animal. Two weeks later, the attachment and the viability of the endometrial explant are examined by a second laparatomy, length, width, and height of the explant are measured and the volume is then calculated. The criterion for a viable graft is fluid accumulation around the lesion. The rate of induction of endometriosis is more than 80%. Animals with endometriosis (with body weights of approximately 250 g) are randomly divided into treatment groups of 10 animals each. Gonadotropin-releasing hormone agonists are injected at doses of 15 or 30 µg/kg subcutaneously daily for 3 weeks. A third laparatomy is performed 3 weeks after the beginning of the experiment. The presence of fluid accumulation and the size of the endometrial explant are examined. The explant is excised and fixed in 10% formalin for histological evaluation.

EVALUATION

The evaluation of treatment on the regression of lesions in experimental endometriosis is analyzed statistically with the χ^2 test.

REFERENCES

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N.9.3
LH-RH antagonistic activity

N.9.3.1
Testosterone suppression in rats

PURPOSE AND RATIONALE

LH-RH antagonists suppress endogenous secretion of LH and testosterone in adult male rats after single subcutaneous injection, and after repeated dosing (Loy 1994).

PROCEDURE

Groups of 8 adult male Wistar rats weighing 200–250 g are injected subcutaneously with various doses of the test compound or a reference LHRH antagonist dissolved in 5% mannitol solution. Four hours later, rats are decapitated and blood is collected from the trunk. Serum is separated in a refrigerated centrifuge (10 min at 3 000 *g*), and stored frozen at –20 °C until assay.

Radioimmunoassay for testosterone

Serum testosterone is measured by radioimmunoassay in serum extracts using a specific antiserum without prior chromatography, or by direct assay in serum depending on the method. Serum samples of 0.5 ml are extracted with 2 ml of freshly purified, peroxide-free diethylether by shaking for 60 s on a Vortex type mixer. The aqueous phase is frozen at –70 °C, the ether phase containing testosterone is transferred to conical test tubes, and evaporated under a stream of dry nitrogen. The dry residue is re-dissolved in BSA/phosphate buffer (1% BSA = bovine serum albumin) for RIA. (1,2,6,7-3H)-Testosterone (New England Nuclear NET 367) and a specific antiserum (AS-781, Behringwerke, Marburg/ Germany) are added and incubated over a period of 24 h at +4 °C under nonequilibrium conditions. Bound hormone and free hormone are separated by adsorption on dextran-coated charcoal by incubation for 30 min at +4 °C and centrifugation at 3 000 *g* for 15 min. 500 µl of supernatant are transferred into minivials and scintillation cocktail is added. Radioactivity is determined in a beta-counter.

EVALUATION

The hormone levels in the sample are calculated from a standard curve by means of a computer program, using appropriate control sera. Using various doses of standard and test preparation dose-response curves can be established allowing calculation of potency ratios with confidence limits. Alternatively, minimum effective doses can be calculated from comparisons with controls. Using different time intervals, e.g., 4, 8, and 24 h, the duration of the effect can be evaluated.

MODIFICATIONS OF THE METHOD

Testosterone suppression can be measured in several animal species, e.g., dogs and marmoset monkeys, or cynomolgus monkeys (Habenicht et al. 1990).

Ayalon et al. (1993) tested the potency of the LH-RH antagonist on the pituitary-gonadal system of female castrated and intact ovulating rats.

Reissmann et al. (1996) investigated the antitumor and hormone-suppressive effect of the LH-RH antagonist Cetrorelix in the model of DMBA-induced mammary carcinoma in female rats and by testosterone determination in normal male rats.

Danzo (1995) studied the effect of a gonadotropin-releasing hormone antagonist on androgen-binding protein production and its distribution among the epididymis, seminiferous tubule fluid, testicular interstitial fluid, and blood in rats.

Fallest et al. (1995) studied the transcriptional regulation of the rat luteinizing hormone β (rLHβ) gene through the use of **transgenic mice** bearing a region of the rLHβ gene linked to a luciferase (LUC) reporter gene. The postgonadectomy rise in pituitary rLHβLUC activity in females and males was blocked by daily administration of the GnRH antagonist Antide.

Rivier et al. (1996) synthesized and evaluated many GnRH analogs and established a dose relationship between GnRH antagonists and pituitary suppression.

Such assays are suitable for initial compounds screening and need to be checked for relevance of the results in intact animals.

REFERENCES

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N.9.3.2 Antiovulatory activity in rats

PURPOSE AND RATIONALE

The rat antiovulatory assay (AOA) was widely used for LHRH antagonists. Immature female rats (24–26 days of age) are primed with pregnant mare serum gonadotropin (PMSG) to induce follicular maturation, followed by spontaneous ovulation 2 days later. The endogenous hypothalamic LH-RH discharge can be blocked by barbiturates (e.g., phenobarbital) to prevent spontaneous ovulation (see N.9.2.10). For anti-ovulatory activity, a test dose of 800 ng exogenous LH-RH is administered together with increasing doses of the LH-RH antagonist. The antagonist will then inhibit the LHRH-induced gonadotropin release. The test measures the dose of LH-RH antagonist required to inhibit the effect of a standard dose of LH-RH.

PROCEDURE

Immature female Wistar rats weighing 55–65 g are injected on day 1 at 9:00 A.M. with 10 IU PMSG subcutaneously. This priming induces follicular maturation and estradiol secretion. A spontaneous, endogenous LH discharge is observed on day 3 between 2:00 and 4:00 P.M. The release of LH and spontaneous ovulation is blocked by i.p. injection of phenobarbital 4 mg/kg on day 3 at 1:00 P.M. The LH-RH antagonist is administered in various doses s.c. or i.p. or i.v. 30 min before phenobarbital injection. Two hours later, a standard dose of 800 ng LH-RH is injected subcutaneously to induce ovulation. Control groups receive phenobarbital only (negative control) or phenobarbital and 800 ng LH-RH (positive control). Six to 8 rats are used for control groups and various doses of the LH-RH antagonist. On the next day, the rats are sacrificed at 9:00 A.M. Both ovaries are prepared and weighed. The oviducts are dissected and stained with Patent blue. The number of ova is counted under a microscope. An effective dose of antagonist prevents LH-RH-induced ovulation.

EVALUATION

A dose-dependent suppression of induced ovulation is observed after the LH-RH antagonist. ID_{50} values can be calculated for various LH-RH antagonists according to the procedure of Litchfield-Wilcoxon. Minimal effective doses which fully suppress ovulation in each animal are calculated.

MODIFICATIONS OF THE METHOD

De la Cruz et al. (1975) described the blockade of the pre-ovulatory LH surge in **hamsters** by an inhibitory analog of LH-RH.

Kovács et al. (1993) found that in ovarectomized and normally cycling rats antiovulatory doses of antagonists of LH-RH inhibit LH and progesterone but not FSH and estradiol release.

Evaluation of biological activities of new LH-RH antagonists in male and female rats was reported by Pinski et al. (1993).

Rivier et al. (1995) tested a series of gonadotropin-releasing hormone antagonists in the rat antiovulatory assay.

Pinski et al. (1995) evaluated the optical isomers of the LH-RH antagonist Cetrorelix in ovulation inhibition in rats and in suppression of LH levels.

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N.9.3.3 Effect of repeated administration of LH-RH antagonists in rats

PURPOSE AND RATIONALE

Repeated administration of LH-RH antagonists reduces the testosterone secretion in serum and tissue content in the testes, and also decreases weight of testosterone dependent organs, such as prostate and seminal vesicles. Moreover, pituitary LH content is decreased and the secretory capacity of testes for testosterone is diminished. The effects are similar to those after supraphysiological doses of LHRH agonists (paradoxical antifertility effects).

PROCEDURE

Male Wistar rats with an initial weight between 150 and 200 g are housed under standard conditions. They are treated with daily injections of the LH-RH antagonist for a period of 7 days up to 4 weeks. Alternatively, the LH-RH antagonist can be administered subcutaneously by infusion via minipumps or other routes of administration. Animals, receiving the vehicle serve as controls, and rats castrated at the beginning of the experiment may be included for maximum inhibition. At the end of the treatment period, the animals are sacrificed 4 h after the last administration. Blood is collected for testosterone determination by radioimmunoassay. The androgen-dependent organs, testes, epididymides, ventral prostate, and seminal vesicles are dissected and weighed to the nearest 0.1 mg. The testes are decapsulated and incubated with 250 mU HCG in order to determine secretory capacity for testosterone. The testosterone tissue content is determined in the supernatant fraction of a testicular homogenate in the absence of HCG (unstimulated testis), and after 3 h of incubation of the contralateral testis with HCG (stimulated testis). Pituitary glands are dissected and the anterior lobe is frozen at –20 °C for determination of LH by rat-specific radioimmunoassay (N.9.2.5). LH receptors in anterior pituitary homogenate are measured by binding of ^{125}I-buserelin *in vitro*.

EVALUATION

The effects of various doses of the LH-RH antagonist after various time intervals on the different parameters mentioned above are compared with values of intact controls and castrated animals. Significant differences vs. controls at 95% level are calculated by Dunnett's test.

MODIFICATIONS OF THE METHOD

These are mainly models for contraceptive applications and tumor suppression using LHRH antagonists.

Kangasniemi et al. (1996) used a combined treatment with a GnRH antagonist and an antiandrogen (flutamide) to suppress spermatogenesis in mice. Despite this effect, the treatment did not enhance recovery from spermatogenesis produced by a 10-Gray dose of radiation.

Hikim and Swerdloff (1993, 1994) examined the time course of suppression and recovery of spermatogenesis and its relationship to the temporal changes in circulating levels of gonadotropin and testosterone and intratesticular testosterone levels after cessation of treatment with a potent GnRH antagonist.

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N.9.3.4 Inhibition of gonadotropin release from anterior pituitary cultures

PURPOSE AND RATIONALE

The same methods as being used for studying the stimulation of gonadotropin release by LH-RH agonists (N.9.2.3, N.9.2.4) can be used for assessment of LH-RH antagonists.

PROCEDURE

Anterior pituitaries of young adult Sprague-Dawley rats are digested with collagenase for 1 h followed by a mechanical dispersion. The resulting cell suspension from 1.5 pituitaries, containing mostly small clusters of cells, is then sedimented together with a suspension of Sephadex G-10 (Sigma) and packed into 6.6-mm columns. Tissue culture medium 199 (Sigma) with supplements, equilibrated with 95% air/5% carbon dioxide, is perfused through the columns at a flow rate of 0.33 ml/min. After an overnight recovery period, during which the baseline stabilizes and the cells regain their full responsiveness, the samples to be tested are introduced through a four-way valve. During a 9 h experimental period, 180 one ml fractions are collected. The system is standardized with 3-min exposures to 100 mM potassium chloride or 3 nM LH-RH. The compounds are introduced in various concentrations, generally for 3–9 min (time of 1–3 fractions), at 30 min intervals. Rat LH levels are measured from aliquots (50 µl) of the collected medium effluent by radioimmunoassay. As a standard, rat LH-RP2 reference standard is used. Repeated stimulation with 3 nM LH-RH for 3 min at 30 min intervals results in a pulsatile LH release.

EVALUATION

The suppression of pulsatile LH release due to repeated administration of LH-RH by antagonists is evaluated.

MODIFICATIONS OF THE METHOD

Krummen et al. (1991) assessed the direct effects of testosterone in primary cultures of pituitary cells.

A special long term superfusion system was developed by Rékási et al. (1993) in order to evaluate the effects of cytotoxic compounds linked to LHRH analogs on different types of rat pituitary cells. LH, GH and PRL were determined simultaneously in the effluent.

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N.9.3.5 Anti-tumor affect of LH-RH antagonists

PURPOSE AND RATIONALE

LR-RH antagonists were found to inhibit experimental tumors in rats and mice.

PROCEDURE

To induce mammary carcinomas, female rats are given a single dose of DMBA (dimethylbenzanthracene)/animal at the age of 50 days. The first mammary tumors can be detected 20–30 days later. The tumor weight is determined by palpation, comparing the volume of each tumor to that of preformed plasticine models. The

tumor weight is calculated by multiplication of the model weight by a factor which takes account the specific weights of plasticine and tumor tissue. After the total tumor mass per animal has reached about 1 g, the animals are randomly divided into treatment groups. At least 8 animals are used per group. The rats are treated for 3 weeks with different subcutaneous doses of LH-RH antagonists or vehicle. The experiment is terminated at the end of the 3rd week from the initiation of the experiment. Histological examination of the tumors is performed.

EVALUATION

The change in tumor volume is calculated on the basis of individual responses. For determination of the mitotic index, 4 000 cells are considered in each tumor.

MODIFICATIONS OF THE METHOD

Reduction of tumor weight in female BDF_1 mice bearing MXT mammary adenocarcinomas after treatment for 3 weeks with a LH-RH antagonist was reported by Szende et al. (1990).

Inhibition of MIA PaCa-2 human pancreatic xenografts in nude mice by a LH-RH antagonist was reported by Radulovic et al. (1993).

The involvement of insulin-like growth factors in growth regulation of the Ishikawa endometrial tumor cell line and the possible interference of LH-RH analogues was evaluated by Kleinman et al. (1993).

Pinski et al. (1994) investigated the effects of treatment with a LH-RH antagonist and a LH-RH agonist in Copenhagen rats bearing the anaplastic androgen-independent Dunning R-3 327-AT-1 prostatic adenocarcinoma implanted orthotopically into the ventral lobes of prostate glands.

Vincze at al (1994) tested the anti-tumor effect of the gonadotropin-releasing hormone antagonist MI-1 544 *in vitro* on human breast cancer lines and *in vivo* on xenografts in immunosuppressed mice.

Manetta et al. (1995) reported the *in vitro* and *in vivo* inhibitory effects of a LH-RH antagonist against a panel of human ovarian carcinomas.

The effects of LH-RH and a LH-RH antagonist on cell growth and production of hCG and cAMP in JAR human chorioncarcinoma cells were examined *in vitro* by Horváth et al. (1995).

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N.9.4 Corticotropin releasing hormone (CRH)

N.9.4.1 General considerations

A hypothalamic factor inducing release of corticotropin (ACTH) from pituitaries was the first of the hypothalamic releasing hormones identified by using *in vitro* bioassays (Saffran and Schally 1955; Guillemin and Rosenberg 1955). A common pre-pro-hormone, opiomelanocortin was identified as the source of corticotropins and endorphins. Much later, the structure of a 41-residue ovine hypothalamic peptide stimulating the secretion of corticotropin and β-endorphin could be identified (Vale et al. 1981), followed by the elucidation of the structure of human CRH (Shibahara et al. 1983) and of other species, such as porcine CRF (Patthy et al. 1985) and equine CRF (Livesey et al. 1991). Reviews are given by Brodish (1979), Rivier and Plotsky (1986), Taylor and Fishman (1988). Conformational differences of ovine and human corticotropin releasing hormone using circular dichroism, Fourier transform infrared spectroscopy, NMR and dynamic light scattering were reported (Dahte et al. 1996).

The binding sites of immunoreactive corticotropin-releasing hormone in the rat ovary and its potential

physiological role were studied by Mastorakos et al. (1993).

Studies on CRF receptors in the pituitary have been performed (Wynn et al. 1983; Millan et al. 1987). Investigations on general pharmacological properties of human corticotropin-releasing hormone did not reveal any considerable side effects (Andoh et al. 1994).

Derivatives with agonistic and antagonistic properties have been synthesized and tested (Rivier et al. 1984; Kornreich et al. 1992; Chen et al. 1996; Schulz et al. 1996; Webster et al. 1996; Arai et al. 1998).

Studies with **CRF antagonists** indicate a role of CRF in certain psychiatric diseases (Schulz et al. 1996) and drug addiction (Koob 1999). This is in line with the known clinical psychotropic effects of corticotropin and glucocorticoids at elevated doses.

The role of corticotropin-releasing hormone in inflammatory processes was investigated by Webster et al. (1998).

Non-mammalian peptides, sauvagine (from frog) and **urotensin** 1 (from fish) have approximately 50% sequence homology with CRF and share *in vitro* and physiological actions characteristic of CRF (Rivier et al. 1983). A mammalian urotensin-like peptide (named **urocortin**) with partial sequence identity with urotensin 1 and CRF has been identified in rat (Vaughn et al. 1995) and human (Donaldson et al. 1996) tissues. The mouse and human urocortin genes have been isolated and characterized (Zhao et al. 1998).

A **corticotropin releasing factor binding protein (CRFBP)** was isolated which is thought to an important modulator of CRF both in the CNS and in the periphery (Behan et al. 1995; Petraglia et al. 1996; Cortright et al. 1997; Hobel et al. 1999). A rise in CRF-BP levels at 30–35 weeks of pregnancy with a dramatic decrease at 38–40 weeks have been shown. *In vitro*, CRF-BP inhibits the ACTH releasing properties of CRF.

Cortright et al. (1995) described a mouse brain corticotropin-releasing hormone-binding protein (CRH-BP) highly homologous to human and rat CRH-BPs, but distinct from the corticotropin-releasing hormone receptor.

Stenzel-Poore et al. (1996) described **transgenic mice** with CRH overproduction inducing behavioral changes that parallel the stress syndrome.

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N.9.4.2 *In vitro* assay for CRH activity

PURPOSE AND RATIONALE

The first *in vitro* assay procedures to detect CRF activity (Saffran and Schally 1955; Saffran et al. 1955) were based on incubation of rat anterior pituitary halves with the test substances in bicarbonate-buffered, oxygenated medium at 38 °C for 1 h in the presence of 0.0004 M DL-arterenol and 0.004 M ascorbate followed by measurement of ACTH release into the medium by incubation with adrenal tissue and determination of corticosterone (a two stage bioassay where a biological product of CRH action is determined by a quantitative biological response). Guillemin and Rosenberg (1955) used pituitary tissue culture from rat and dog and determined the ACTH activity in the medium by the *in vivo* ascorbic acid depletion method in the rat according to Sayers (1954) (see N.7.4.1). The assay was later considerably simplified by using ACTH RIA. Several modifications of these procedures have been used (Schally et al. 1968; Yasuda et al. 1982). Most investigators used rat pituitary cell cultures (Giguère and Labrie 1982; Giguère et al. 1982; Bilezikjan and Vale 1983; Aguilera et al. 1983; Vale et al. 1983a,b; Patthy et al. 1985), while pituitary segments have been used by Antoni et al. (1983) and by Widmaier and Dallman (1984).

PROCEDURE

Anterior pituitaries are obtained from adult Sprague-Dawley rats, cut into small pieces and incubated in a Dubnoff incubator for 45 min at 37 °C in 10 ml of oxygenated Medium 199 (GIBCO) containing 0.5% collagenase, 0.25% BSA, and 50 µg/ml gentamycin. The fragments can be easily dispersed mechanically into single cells by repeated suction and expulsion from a pipette. The cell suspension is centrifuged at room temperature for 10 min at 100 *g*. The cell pellet is then resuspended in 1.0 ml of medium and divided into 4 equal volumes. Each volume (containing about 5×10^6 cells) is mixed with 0.5 ml Sephadex G-15 which has been equilibrated previously with oxygenated medium. The mixture of pituitary cells and Sephadex is transferred into 4 chambers of a superfusion apparatus (Vigh and Schally 1984; Czernus and Schally 1991) consisting of a number of 1 ml plastic syringe barrels (modified by cutting off their distal end) mounted vertically in a Plexiglas holder which is kept at 37 °C by circulating water. Each barrel is fitted with plungers at both ends. Holes are drilled in the plungers to accommodate plastic tubing. The lower plunger is covered with a small piece of 30 µm-pore nylon net

to keep the Sephadex beads from escaping. The "pores" between the beads are small enough to prevent the pituitary cells from escaping and large enough to allow unrestricted flow of medium through the column. The upper plunger tubing is used for directing the flow through the chamber from the medium reservoir. The flow through the system is controlled by a multichannel peristaltic pump which is placed after the superfusion chamber. Thus, the system is operated by suction with negative pressure.

The cells are perfused overnight with medium 199 (Sigma) at a flow rate of 20 ml/h. The samples are administered every half h for 3 min and 1-ml fractions are collected at 3-min intervals. Synthetic CRF at 0.2 and 2 mM is used as standard. The corticotropin released into the medium is measured by a specific RIA for corticotropin.

EVALUATION

Dose-response curves are established for test preparation and standard allowing calculation of potency ratios with confidence limits.

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N.9.4.3 *In vivo* bioassay of CRH activity

PURPOSE AND RATIONALE

Munson and Briggs (1955) described an assay using morphine and pentobarbital pretreated rats. Many modifications for a reliable *in vivo* bioassay of CRH activity have been tested including rats with hypothalamic lesions (Schally 1968; Yasuda et al. 1982). Arimura et al. (1967) described an assay for corticotropin-releasing factor (CRF) using rats treated with morphine, chlorpromazine, dexamethasone and nembutal. This assay has been further improved by Graf et al. (1985).

PROCEDURE

Male Sprague-Dawley rats weighing about 200 g are maintained on a 12:12 h light:dark cycle at constant temperature for at least 5 days before use. On the day of the experiment, the rats are prepared with an initial single subcutaneous injection of a mixture of chlorpromazine (10 mg/kg) and morphine sulfate (20 mg/kg) followed after 75 min by pentobarbital (25 mg/kg) i.p. Sixty min after the injection of pentobarbital, various doses of test substances or standard or vehicle (0.9% NaCl, 1% BSA, 0.1% ascorbic acid) are injected into an exposed jugular vein. Blood samples (0.5 ml) are collected in heparinized syringes immediately before and 30 min after injection of test substances. The

blood is transferred to chilled polypropylene tubes containing EDTA (1.0 mg) and aprotinin (Trasylol®, 50 µl) and centrifuged (2 000 *g*, 15 min 4 °C). Plasma samples are stored at –20 °C for corticosterone assay using a crossreacting cortisol RIA that also measures corticosterone, the main corticoid secreted by rats, or a corticosterone RIA.

EVALUATION

Data for CRH activity of experimental groups are evaluated by analysis of variance or covariance followed by Duncan's multiple range test.

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N.9.4.4 Collection of hypophyseal portal blood in rats

PURPOSE AND RATIONALE

Worthington (1966), Porter and Smith (1967), Gibbs (1984), Sarkar and Minami (1991) described methods to collect hypophyseal portal blood in rats. These methods can be used to measure the changes in secretion of several hypothalamic hormones under the influence of various factors.

Gibbs and Vale (1982), Gibbs (1985a), Plotsky and Sawchenko (1987) studied the effect of various conditions on hypophyseal portal plasma levels of CRF and arginine vasopressin.

Luteinizing hormone-releasing hormone (LHRH) was determined by Ching (1982), Sarkar (1987), Petraglia et al. (1987),

thyrotropin releasing hormone (TRH) by Fink et al. (1982),

growth hormone-releasing hormone (GHRH) by Plotsky and Vale (1985),

β-endorphin by Sarkar and Yen (1985),

neuropeptide Y by Sutton et al. (1988),

vasoactive intestinal peptide by Sarkar (1989),

vasopressin and oxytocin by Gibbs (1984),

dopamine and epinephrine by Ben-Jonathan et al. (1977), Gibbs (1985).

PROCEDURE

Pentobarbital anesthetized rats are placed on an isothermal heating pad in a stereotactic instrument. After cannulation of the trachea, the animals are mechanically ventilated. The base of the skull is exposed by a transpharyngeal approach, and the basosphenoid bone is drilled away. The dura is cut and deflected, the rat is heparinized, and after the stalk is cut, a polyethylene cannula filled with saline is placed over the hypophyseal stalk for collection of blood. The cannula leads to an ice bath, and the distal end of the tubing ends in a fraction collector where portal blood is collected by gravity flow at a rate of about 10 µl/min.

EVALUATION

Portal plasma concentrations of CRH, LHRH, TRH, GHRH, β-endorphin, neuropeptide Y, vasoactive intestinal peptide, vasopressin and oxytocin and catecholamines were determined after various treatments and compared with control periods of spontaneous secretion.

MODIFICATIONS OF THE METHOD

Cowell et al. (1991) described *in vitro* models for the examination of the mechanisms controlling the secretion of hypothalamic hormones.

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N.9.4.5 CRH receptor determination

PURPOSE AND RATIONALE

CRH mediates its effects through high-affinity receptors identified and characterized by radioreceptor studies, cellular signaling was measured by stimulation of adenyl cyclase in membrane fractions of rat brain, pituitary and spleen (Grigoriadis et al. 1993). Cloning studies indicate the existence of at least two types of mammalian receptor (De Souza et al. 1998). The CRF_1 receptor has been cloned from several species including human (Chen et al. 1993; Castro et al. 1996; Di Blasio et al. 1997; Grammatopoulos and Hillhouse 1998), mouse (Vita et al. 1993), and rat (Perrin et al. 1993). Species homologies are 98% identical over the full length of 415 amino acids.

There are three isoforms of the CRF_2 receptor: The $CRF_{2(a)}$ receptor which has been cloned from both rat (Lovenberg et al. 1995) and human (Liaw et al. 1996), is a 411 amino acid protein which shares approximately 70% identity with the CRF_1 receptor. The $CRF_{2(b)}$ receptor isoform has been cloned from rat (Lovenberg et al. 1995), mouse (Stenzel et al. 1995) and human (Kostich et al. 1996) and is 431 amino acids in length, differing from the $CRF_{2(a)}$ receptor in that the first 34 amino acids in the N-terminus are replaced by 54 different amino acids. The $CRF_{2(c)}$ receptor has been identified in human brain (Sperle et al. (1996).

The [^{125}I]sauvagine binding to CRH_2 receptors has been characterized by Rominger et al. (1998).

CRH in plasma is mostly bound to the corticotropin-releasing hormone-binding protein (CRH-BP) and is therefore inactive except for the free fraction. CRH-BP is predominantly produced by the liver and distributed and expressed differently from the CRH receptors (Cortright et al. 1995; Zhao et al. 1997).

Rhode et al. (1996) used whole brains of Wistar rats for the CRH receptor assay.

PROCEDURE

Whole brains of male Wistar rats weighing 220–250 *g* are homogenized with a Teflon-glass homogenizer (10 strokes at 800 rpm) in 0.32 M sucrose, 50 mM Tris/HCl (pH 7.2), 10 mM $MgCl_2$, 2 mM EGTA, and 0.15 mM bacitracin at 50 mg wet weight per ml. After centrifugation at 1 000 *g* for 5 min, the supernatant is centrifuged at 26 000 *g* for 20 min. The pellet is resuspended in 50 mM Tris/HCl (pH 7.2), 10 mM $MgCl_2$, 2 mM EGTA, adding 0.15 mM bacitracin and 0.0015% aprotinin (assay buffer) and again centrifuged. The pellet is suspended in assay buffer containing 0.32 M sucrose and stored at –20 °C. All steps are carried out at 4 °C. Protein concentrations are determined by the method of Bradford (1976) using BSA as standard.

One hundred µg of membrane preparation in 300 µl assay buffer are incubated in quadruplicate with 0.1 nM [^{125}I]Tyr-oCRH in the absence and presence of 12 different concentrations (0.2 nM up to 1 µM) of unlabeled peptides at 25 °C for 2 h. Nonspecific tracer binding is determined in the presence of 1 µM oCRH. At the end of incubation, 3 ml of ice-cold (assay buffer with-

out inhibitors containing 0.01% Triton X-100) is added to the assay tube and the samples are immediately filtered through GF/C filter discs (Whatman), presoaked for 2 h in 0.1% polyethylenimine using a Brandel-Harvester. The incubation tubes and filters are then washed with 3 ml cold washing buffer. Triton X-100 in this buffer strongly reduces nonspecific tracer binding. Radioactivity retained on the filter is measured by γ-counting.

EVALUATION

Receptor affinities (Kass, $K_d = 1 / \text{Kass}$) and capacities (B_{max}) are estimated using the nonlinear least-squares curve fitting program RADLIG (BIOSOFT, Cambridge, UK) and a K_d of 0.48 nM for the binding of the tracer peptide as determined from tracer saturation curves.

MODIFICATIONS OF THE METHOD

Schulz et al. (1996) used P2 membranes from human neuroblastoma IMR32 cells to test receptor binding of a selective nonpeptide antagonist of corticotropin releasing factor.

Webster et al. (1996) used rat frontal cortex, pituitary, cerebellum, and heart tissue for *in vitro* characterization of antalarmin, a nonpeptide CRH receptor antagonist.

Differences between normal and adenomatous pituitary corticotrophs in labeling characteristics of corticotropin-releasing hormone receptors were reported by Abs et al. (1997).

Rabadan-Diehl et al. (1997) studied the role of glucocorticoids and hypothalamic factors in regulation of pituitary corticotropin releasing hormone receptor mRNA and CRH during adrenalectomy in rats.

The differential regulation of hypothalamic pituitary corticotropin releasing hormone receptors during development of adjuvant-induced arthritis in rats was reported by Aguilera et al. (1997).

Grammatopoulos and Hillhouse (1998) reported the solubilization and biochemical characterization of the human myometrial corticotrophin-releasing hormone receptor.

The interaction between glucocorticoids and corticotropin releasing hormone in the regulation of the pituitary CRH receptor has been studied in rats by Ochedalski et al. (1998).

The multiple actions of corticotropin-releasing factor on neuroendocrine and behavioral functions were examined using high affinity, non-peptide antagonists (Lundkvist et al. 1996).

Wei et al. (1998) described analogs of CRH and urocortin with selective activity at CRH_1 and $CRH_{2\beta}$ receptors.

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N.9.5
Growth hormone releasing hormone (GH-RH)

N.9.5.1
Radioreceptor assay of growth hormone-releasing hormone

PURPOSE AND RATIONALE

As for other polypeptide hormones, GHRH receptors may be used for quantification and potency estimates of relative binding affinities. Several agonistic and antagonistic analogs of human growth hormone-releasing hormone (hGH-RH) have been synthesized by various groups of investigators. The clinical and basic aspects of growth hormone-releasing peptides have been reviewed by Argente et al. (1996).

In vitro assays with pituitary cells have been used for screening biological activity of hGH-RH analogs prior to their evaluation *in vivo* (Campbell et al. 1991; Heiman et al. 1985; Kovacs et al. 1988). A careful determination of binding activities of the peptides to specific GH-RH receptors provides important data for the design of more active analogs. Several studies demonstrated that the radioligand, [His^1-,^{125}I-Tyr^{10},Nle^{27}]-hGH-RH(1-32)NH_2 binds specifically to a single class of receptors in rat pituitary cells and homogenates, and can be used for radioreceptor assay (Campbell et al. 1991; Seifert et al. 1985a,b; Struthers et al. 1989). The use of a radioreceptor assay for *in vitro* screening of analogs of growth hormone-releasing hormone was described by Halmos et al. (1993).

PROCEDURE

Pituitaries from male Sprague Dawley rats (250–300 g) are used to prepare crude membranes. Immediately after decapitation, anterior pituitaries are removed, rinsed with cold saline, and homogenized in 5 times their volume of homogenization buffer (50 mM Tris-HCl, 5 mM EDTA, 5 mM $MgCl_2$, 30 µg/ml bacitracin, pH 7.4) on ice using an Ultra-Turrax homogenizer at maximal speed. The homogenate is centrifuged at 500 *g* for 10 min at 4 °C. The supernatant containing the crude membrane fraction is again centrifuged at 70 000 *g* for 50 min at 4 °C. The pellet is washed twice by resuspending in ice-cold homogenization buffer and spinning. The final pellet is resuspended in homogenization buffer and stored at –70 °C until used for the receptor binding studies. Protein concentration is determined by the method of Bradford (1976) using a commercially available protein assay kit.

[His^1,^{125}I-Tyr^{10},Nle^{27}]hGH-RH(1-32)NH_2 is iodinated by the chloramine-T method (Greenwood et al. 1963). The receptor binding assay is carried out for

60 min in GH-RH binding buffer (50 mM Tris-HCl, 5 mM EDTA, 5 mM $MgCl_2$, 1% BSA, 30 µg/ml bacitracin, pH 7.4) at 24 °C. For saturation binding analyses, 50–150 µg membrane homogenates are incubated in duplicate with a least 6 concentrations of radio-ligand, ranging from 0.005 to 0.35 nM in the presence or absence of excess unlabeled peptide (1 µM) in a final volume of 300 µl. Incubation is terminated by immersing siliconized borosilicate tubes in ice water, transferring 270 µl of the suspension into cold siliconized polypropylene microfuge tubes, and centrifuging at 12 000 *g* for 2 min at 4 °C, and aspirating the supernatants. To reduce the nonspecific binding, 500 µl/tube of GH-RH binding buffer is added and the tubes recentrifuged. The washing step is repeated and the supernatant again aspirated. Finally, the bottoms of the tubes, containing the pellet, are cut off and counted for radioactivity in a gamma-counter.

In the competition experiments, which also include the specificity experiments, 50–100 µg of membrane homogenates are incubated in duplicate with 0.10–0.15 nM radioligand plus various concentrations of nonradioactive analogs and other peptides (10^{-12}–10^{-6} M).

EVALUATION

Percent specific binding is plotted against the log concentration of competitors. The curves are compared at the 50% specific binding levels (IC_{50}). The ligand-PC and McPherson computerized curve fitting programs of Munson and Rodbard (1980) are used to analyze competition and saturation data. To determine the types of receptor binding, dissociation constants (K_d) and the maximal binding capacity of receptors (B_{max}), the saturation binding data are also analyzed by the Scatchard method.

MODIFICATIONS OF THE METHOD

Carrick et al. (1995) described a rapid and sensitive binding assay for growth hormone releasing factor. Human embryonic kidney (HEK293) cells and rat pituitary tumor (GH_4C_1) cells were transfected with the porcine GRF receptor cDNA. Stably expressing cell lines are referred as 293-P2 and GH_4-P1, respectively. GH_4C_1 cells transfected with somatostatin receptor subtype 2 are called GH_4-R2.20 and HEK293 cells transfected with somatostatin receptor subtype 5 are called 293-R5.2. GH_4-P1 and 293-R5.2 were derived by the calcium phosphate method of transfection and 293-P2 and GH_4-R2.20 using lipofectamine. For binding assays, cells were removed from culture dishes by the addition of phosphate buffered saline containing 1 mM EDTA and cell membrane fractions prepared. Membrane pellets were suspended in 25 mM Tris, pH 7.4, plus protease inhibitors, aliquoted and stored at –80 °C. The binding assay consisted of approximately 25 pM radiolabeled ligand in the presence or absence of unlabeled ligand in assay buffer (50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 2 mM EGTA) and cell membranes (2–10 µg). The total assay volume of 200 µl included wheat germ agglutinin coated SPA beads at a concentration of 1 mg/assay, and for GRF binding, 0.05 mg/ml alamethicin (Sigma St. Luis, USA). Ninety-six well plates were used for most assays. Assays were agitated on a plate shaker for 3 h. Samples were counted on a Wallac Microbeta Counter for 1 min per sample. The conventional binding assay was performed (Mayo 1992), except that following binding and centrifugation the membranes were washed in 0.5 ml binding buffer, centrifuged and counted.

Abribat et al. (1990), Gaudreau et al. (1992) developed a binding assay for GH-RH in rat pituitaries. Using this technique, Lefrancois and Gaudreau (1994) tried to identify the receptor-binding pharmacophores of GHRH in rat pituitaries.

Hassan et al. (1995) developed a competitive binding assay using cloned porcine growth hormone-releasing hormone receptors in order to study structure-activity relationships.

Kaijkowsi et al. (1997) investigated growth hormone releasing hormone receptor structure and activity using yeast expression technologies.

Muccioli et al. (1998) characterized specific binding sites for synthetic GH secretagogues (sGHS) on membranes from pituitary gland and different human brain regions using a peptidyl sGHS (Tyr-Ala-hexarelin) which has been radio-iodinated to high specific activity at the Tyr residue.

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N.9.5.2 Growth hormone release from rat pituitaries *in vitro*

PURPOSE AND RATIONALE

For testing of pituitary cell stimulation, isolated pituitary glands or cell cultures may be used. Antagonism by adding e.g. somatostatin is tested in the presence of a standard concentration of GHRH. Human GHRH has the structure of a 44-amino acid amide, which was isolated from human endocrine tumors and later confirmed as a hypothalamic hormone. The effect of GHRH analogues can be tested on isolated rat pituitaries measuring GH release. This test systems avoids the interference of counterregulatory somatostatin secretion which limits the duration of GH release *in vivo*.

PROCEDURE

The pituitaries of male Sprague-Dawley rats weighing about 100 g are quickly removed after decapitation. The posterior lobe is discarded and the anterior lobe is divided into two halves by a midsagittal cut. Five bisected hemipituitaries are incubated in plastic vials containing 4 ml TCM 199 with 0.1% BSA, 15 µg/ml penicillin and 25 µg/ml streptomycin. The vials are gassed with 95% O_2 and 5% CO_2. After 30 min of control incubation, the medium is changed and various doses of standard and test substances are added for an incubation of 90 min. GH content in the medium and in the pituitaries are determined by a specific radioimmunoassay (Schalch and Reichlin 1966).

EVALUATION

Dose-response curves are established for standard and test compounds measuring GH release into the medium and GH depletion from the pituitaries allowing calculation of potencies ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Superfused pituitary cells may be used measuring activity and duration of effect, as well as interaction of stimulatory and inhibitory factors. Growth hormone releasing factor from tumors in human pancreas and from rat hypothalami as well as analogs of growth hormone-releasing hormone were evaluated in a superfused pituitary cell system (Vigh and Schally 1984; Czernus and Schally 1991; Halmos et al. 1993), see also Sect. N.9.4.2, *in vitro* assay for CRH activity. Anterior pituitaries of two young adult male Sprague Dawley rats were digested with 0.5% collagenase CLS2 (Worthington) for 50 min. After incubation, the fragments were digested into cell clusters (5–40 cells) by mechanical dispersion, and then transferred onto two columns and allowed to sediment simultaneously with 0.8 ml Sephadex G-10. The dead volume of the system was set to 1 ml. Medium 199 containing BSA (2.5 g/l), $NaHCO_3$ (2.2 g/l) and gentamycin sulfate (85 µg/ml) was equilibrated with a mixture of 95% air and 5% CO_2 and used as the culture medium. The medium was pumped at a flow rate of 0.33 ml/min. During an overnight recovery period, the baseline stabilized and the cells regained their full responsiveness. The samples were then infused through a four-way valve at 5×10^{-10} M concentration for 3 min (1 fraction) at 45-min intervals. Rat GH was determined by double-antibody radio-immunoassay.

The same system was used by Rekasi and Schally (1993) and Kovács et al. (1996a) to evaluate the activity of growth hormone-releasing hormone antagonists. For determination of the antagonistic activity, the cells were exposed to 10^{-8}, 10^{-7}, and 10^{-6} M GHRH antagonist simultaneously with 10^{-9} M GHRH or to 10^{-6} M

GHRH antagonist combined with 100 mM KCl (controls for potassium stimulated GH secretion) for 3 min. After 30 min, the duration of the inhibitory effect of GHRH antagonist was also tested by repeated 3-min infusions of 10^{-9} M GHRH.

Using this system with pituitaries of **transgenic mice** overexpressing the human GHRH gene, Kovacs et al. (1997) evaluated the effects of growth hormone-releasing hormone antagonists.

In addition to growth hormone release, Horváth et al. (1995) determined cAMP release from superfused rat pituitary cells stimulated by growth hormone-releasing hormone.

GH release was determined using cultured rat pituitary cells (Brazeau et al. 1982; Perkins et al. 1983; Scheikl-Lenz et al. 1985). Pituitary cells were prepared by enzyme dispersion with collagenase, DNAase and pancreatin. The cells were cultured for 3 days in microbiological Petri dishes in Dulbecco's modified essential medium with 20 mM HEPES, 15% fetal calf serum, 100 mU/ml penicillin-G and 100 µg/ml streptomycin at 37 °C and 10% CO_2.

Cheng et al. (1993) tested time- and dose-dependent growth hormone release by a non-peptidyl growth hormone secretagogue in rat pituitary cells.

Sanchez-Hormigo et al. (1998) tested growth hormone-releasing hexapeptide, one of several synthetic GH secretagogues, on growth hormone secretion from cultured porcine somatotropes.

Kovács et al. (1996b) measured the effects of chronic administration of a growth hormone-releasing hormone agonist on body weight, tibia length and tail length in growth hormone-deficient (monosodium glutamate-lesioned) rats.

Jacks et al. (1996) evaluated an orally active growth hormone secretagogue in dogs. Serum growth hormone levels were dose-dependently increased after oral and intravenous administration. Moreover, an increase of insulin-like growth factor and serum cortisol was found.

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N.9.5.3
GH-RH bioassay by growth hormone release in rats

PURPOSE AND RATIONALE

Releasing activity can be determined by plasma GH stimulation after intravenous or subcutaneous injection of GH-RH in rats.

PROCEDURE

Male Sprague-Dawley rats weighing about 100 g are anesthetized by fractionated subcutaneous injections of 0.7 ml/kg of 25% urethane solution. The jugular vein is cannulated. Various doses of GH-RH test preparations or standard are injected intravenously in 0.2 ml 1% gelatine/saline. Fifteen min later blood samples are collected for determination of GH by a specific radioimmunoassay (Schalch and Reichlin 1966). The plasma concentration is expressed in terms of NIAMD-rat-GH-RP-1.

As a modification also suitable for other releasing hormones tested e.g. by subcutaneous application, a double-barreled polyethylene catheter is implanted into the jugular vein, one lumen being used for blood sampling, the second small lumen being used to keep the catheter open by heparin infused into the tip of the collection catheter. Blood is continuously withdrawn by a peristaltic pump and fractions of 500 µl heparinized blood are collected at intervals of 10 min. To compensate for blood losses, the erythrocyte fraction of 4–8 consecutive plasma samples is re-infused in a plasma expander (Haemaccel® 3.5%) via a femoral vein. Before treatment, two 2 min samples are collected, and after injection, two 2 min samples are collected again, followed by sampling intervals of 10 min for up to 3 h. GH is determined by a specific radioimmunoassay (Schalch and Reichlin 1966).

EVALUATION

Using several doses of standard and test preparation dose-response curves are established allowing calculation of potency ratio with confidence limits. Time concentration curves after subcutaneous or e.g. intranasal application allow to evaluate the duration of action.

MODIFICATIONS OF THE METHOD

Wehrenberg and Ling (1983), Wehrenberg et al. (1985) determined *in vivo* biological potency of rat and human growth hormone-releasing factor and fragments of human growth hormone-releasing factor in swivel-cannulated conscious freely moving rats and in cannulated anesthetized rats.

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N.10
Other peptide hormones

N.10.1
Melanophore stimulating hormone

N.10.1.1
Skin darkening in whole amphibia

PURPOSE AND RATIONALE

MSH (Melanocyte stimulating hormone or melanotropin) is also called melanophore expanding hormone based on the microscopical observation of expansion of melanocytes in amphibia. Several amphibia species can be used, such as *Xenopus laevis*, *Rana temporaria* or *esculenta*, or *Hyla arborea*. Hypophysectomized animals are more sensitive than intact ones. Numerous corticotropin analogs have MSH activity based on a common peptide core shared with MSH. The structure of two melanocyte-stimulating hormones has been elucidated: α-MSH containing 13 amino acids, and β-MSH containing 18 amino acids.

Hunt (1995) reviewed the role of melanocyte-stimulating hormone as a regulator of human melanocyte physiology.

PROCEDURE

The pretest conditions require adaptation for blanching of the skin, e.g., by keeping the animals on a white background prior to the assay. *Xenopus laevis* or *Rana temporaria* are kept in single cages at 16 °C at humid conditions. Injections of various doses of the test preparation or the standard are given into the dorsal lymph sac. One hour later, the webs between the digits of the hind limbs are investigated under a stereomicroscope, the eyepiece of which is fitted with a photoelectric cell, the other being available for microscopic examination. The state of melanophore expansion can be assessed by direct visual examination and recorded in terms of an arbitrary melanophore index, or by modern methods of morphometry.

EVALUATION
For test preparation and standard, dose-response curves can be established and potency ratios calculated.

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N.10.1.2 Assay in isolated amphibian skin

PURPOSE AND RATIONALE
Melanophores in isolated pieces of pale amphibian skin (from background adapted animals or maintained in MSH free medium) expand within a short period of time after immersion in a MSH-containing buffer. Subsequent immersion of the skin in fresh saline results in contraction of the melanophores (Trendelenburg 1926; Jores 1933). Several methods using isolated amphibian skin have been described using either light absorption or light reflection (Landgrebe and Waring 1962). These methods are historical and have been replaced by using cell cultures of melanocytes.

PROCEDURE
For studies on light absorption the skin of *Rana temporaria* or *Rana esculenta* is used. Before sacrificing the donor animal and dissecting the isolated skin, it is important to submit the living animal to varying white and black backgrounds under overhead illumination of a light source of 100 W, so that the melanophores can expand and contract before operation. The areas most suitable for using in this technique are from the thighs. Before using the skin for an actual assay, each piece should be immersed in a sufficiently concentrated MSH-solution to expand the melanophores almost but not quite fully. The skin is then washed with saline until the melanophores are fully contracted again and in a suitable condition for the test. This usually takes about 45 min. A piece of skin is mounted in a cell which holds the skin stretched and enables it to be immersed in saline or test solution. It is placed in position on the stage of a binocular microscope. An eyepiece is used for visual observation and a photoelectric cell is attached to the other connected directly to a sensitive galvanometer. Various concentrations of the test preparation or the standard are added. At least 6 skin pieces are used for each concentration of test preparation or standard. Galvanometer readings and readings of the melanophore index are taken simultaneously every 10–15 min until the maximum response is elicited, usually in 30 to 45 min.

EVALUATION
Assessment of potency is made from the readings of the melanophore index and from the light absorbed which is recorded as a percentage of the light originally passing through the skin and is plotted against the log concentration.

CRITICAL ASSESSMENT OF THE METHOD
The methods were originally developed to standardize extracts with MSH-activity by the use of a bioassay. They are still necessary to evaluate compounds for biological MSH-activity since not only natural ACTH but also synthetic peptides, such as β^{1-24}-corticotropin (Schuler et al. 1963) or β^{1-23}-corticotropin-23-amide (Vogel 1965, 1969), possess MSH-activity.

MODIFICATIONS OF THE METHOD
Measurement of light reflection of skin pieces instead of light absorption has been used by Shizume et al. (1954).

In **cell-based assays**, Siegrist and Eberle (1986), Bagutti and Eberle (1993, Sahm et al. 1993, 1966) used cultured mouse B16 melanoma cells in a sensitive *in situ* melanoma assay to study structure-activity of melanocyte-stimulating hormone peptides. B16 Cells were seeded at a density of 2 500 cells per well in 96-well microtest culture plates. After 24 h the cells were incubated in the presence of serial dilutions of MSH peptides for 3 to 5 days. The melanin released into the medium of each well was then determined spectrophotometrically at a wavelength of 405 nm using an automatic microplate reader calibrated against synthetic melanin.

Sahm et al. (1994, 1996) measured release of ^3H_2O into the medium from [3',5'-^{3}H]L-tyrosine by tyrosinase in B16 mouse melanoma cells after incubation with α-MSH analogues.

MSH assay have also been used to study the factors regulating the release of MSH (**MRF**) or inhibiting the release of MSH (**MIF**) (Kastin et al. 1969; Celis et al. 1971).

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N.10.1.3 Binding to the melanocortin receptor

PURPOSE AND RATIONALE

Much of the initial work on biological effects of hormones has been expanded by using cloned subclasses of receptors for identification of more specific ligand compounds. A family of five MSH receptor subclasses for the melanocortic peptides has been identified (Chhajlani and Wikberg 1992; Chhajlani et al. 1993; Gantz et al. 1993b; Schiöth et al. 1988a; Strand 1999). The melanocortin MC_1 receptor is expressed in melanocytes and melanoma cells and binds α-MSH with high affinity. The MC_1 receptor plays an important role in skin and fur pigmentation in a variety of vertebrates (Cone et al. 1996). The melanocortin MC_2 receptor (i.e., the ACTH receptor) has a well-defined function of the steroid production in the adrenal gland (Schiöth et al. 1996a). The melanocortin MC_3 receptor is found in the hypothalamus, the brain and in the placenta, gut tissues and the heart (Gantz et al. 1993a; Sahm et al. 1994; Desarnaud et al. 1994). The melanocortin MC_4 receptor (Schiöth et al. 1996b, 1998b) has been found to affect feeding in rodents and may be important for weight homeostasis (Fan et al. 1997; Huszar et al. 1997). The melanocortin MC_5 receptor is primarily located in various peripheral tissues but has also been found in the brain (Labbé et al. 1994; Fathi et al. 1995).

Schiöth et al. (1998a) described binding of synthetic MSH analogues to the human melanocortin receptor subtypes.

Melanocortin-4 receptor antagonists increase food uptake in rats (Kask et al. 1998a,b; Skuladottir et al. 1999).

PROCEDURE

Expression of receptor clones

The human melanocortin MC_1 and human melanocortin MC_5 receptors are cloned into the expression vector pRc/CMV (In vitrogen). The human melanocortin MC_3 and human melanocortin MC_4 receptors were cloned into the expression vector pCMV/neo. For receptor expression COS-1 cells are grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Eighty-percent confluent cultures are transfected with the DNA mixed with liposomes in serum-free medium. After transfection, the serum-free medium is replaced by serum-containing medium and the cells are cultivated for 48 h. Cells are then scraped off, centrifuged and used for radioligand binding.

Binding studies

The transfected cells are washed with binding buffer (Schiöth et al. 1995) and distributed into 96-well non-culture-coated plates, which are centrifuged and the binding buffer is removed. The cells are then immediately incubated in the well plates for 2 h at 37 °C with 0.05 ml binding buffer in each well containing a constant concentration of [125I]NDP (= [Nle^4-D-Phe^7]α-MSH) and appropriate concentrations of the competing unlabeled ligand. After incubation the cells are

washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 M NaOH. Radioactivity is counted by a gamma-counter.

EVALUATION

Data are analyzed by fitting to formulas derived from the law of mass action. The method is generally referred to as computer modelling. K_d values for [125I]NDP are calculated (Schiöth et al. 1995, 1996a). The binding assays are performed in duplicate and repeated three times.

MODIFICATIONS OF THE METHOD

Alan et al. (1994) compared the behavioral effects of melanocortins with binding data on MC_3 and MC_4 receptors.

Sahm et al. (1996) studied receptor binding affinities and biological activities of linear and cyclic melanocortins in B16 murine melanoma cells expressing the native MC_1 receptor.

Quillan and Sadée (1996) searched for peptide ligands that cross-react with melanocortin receptors and found several peptides with previously unrecognized agonistic or antagonistic activity on amphibian and human melanocortin receptors.

Peng et al. (1997) compared the actions of C-terminally modified melanocortin peptides at rodent MC_1 and MC_3 receptors.

Bagutti et al. (1993) recommended [^{111}In]DTPA (diethylenetriaminopentaacetic acid)-labeled analogues of α-MSH as ligands for the detection of MSH receptors *in vitro* and *in vivo*.

Erskine-Grout et al. (1996) described functional photoaffinity-labeled, biotinylated and fluorescent probes for the melanoma MC_1 receptor.

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N.10.2 Relaxin

N.10.2.1 General considerations

Relaxin is a peptide hormone originally prepared from sow ovaries. The hormone acts on collagen and induces increased flexibility of the pelvic girdle of guinea pigs. In mice, the length of interpubic ligament is increased. Moreover, relaxin inhibits spontaneous uterine motility. In addition to its effects on the reproductive tract, relaxin exerts hemodynamic effects (Coukson et al. 1996). The actions of relaxin as a pleiotropic hormone were reviewed by Bani (1997).

The therapeutic relevance of relaxin in man is still open to debate (Schwabe and Büllesbach 1994; Goldsmith et al. 1995).

The primary structure of relaxin is highly homologous to insulin. Unlike insulin, the structure of which is remarkably well conserved among the vertebrates, relaxin sequences can vary by more than 50% between different species. Despite these large variations, most relaxins have very similar biological activities in animal test systems, probably, because the receptor binding region of the B chain, in contrast to the rest of the molecule, is highly conserved between species.

Relaxin of various species has been synthesized (Büllesbach and Schwabe 1993; Wade et al. (1994).

Synthetic human relaxin has been characterized by high-performance liquid chromatography (Canova-Davis et al. 1990).

Klonisch et al. (1999) determined the nucleic acid sequence of canine **preprorelaxin** using reverse transcription- and rapid amplification of cDNA ends-polymerase chain reaction. Canine preprorelaxin consists of 534 base pairs encoding a protein of 177 amino acids with a signal peptide of 25 amino acids, a B domain of 35 amino acids, a C domain of 93 amino acids, and an A domain of 24 amino acids.

The **relaxin-like factor (RLF)** is described as a member of the insulin/relaxin/insulin-like growth factor family that is expressed predominantly in the reproductive system, with highest expression in the Leydig cells of the testis (Pusch et al. 1996; Zarreh-Hoshyari-Khah et al. 1999).

Several **bioassays** have been used for relaxin, such as the pubic symphysis method in guinea pigs and mice, inhibition of uterine motility in rats, stimulation of interstitial collagenase activity in cultured uterine cervical cells from guinea pigs (see below).

Taylor and Clark (1989, 1992a,b) developed a **reverse hemolytic plaque assay** which allows the quantitative analysis of relaxin secreted by single porcine luteal cells.

Radioimmunoassays for relaxin were described by Sherwood (1979), Jockenhövel et al. (1991, Steinetz et al. 1996). Lucas et al. (1989) developed an enzyme-linked immunoassay to study human relaxin in human pregnancy and in pregnant rhesus monkeys.

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N.10.2.2 Relaxin bioassay by pubic symphysis method in guinea pigs

PURPOSE AND RATIONALE

Experimental relaxation of the pubic symphysis of the spayed guinea pig was described as early as 1929 by Hisaw. The relaxation of the symphysis after relaxin administration is determined by manual palpation.

PROCEDURE

Virgin female guinea pigs of mixed strains weighing 300–400 g are used. Prior to the assay, the animals have to be primed with estrogen and relaxin. Estrogen priming consists of one subcutaneous injection per week of 5 µg of estradiol cyclopentylpropionate in 0.1 ml of sesame oil. Relaxin priming is accomplished by administering 20 µg of relaxin standard in 1 ml saline subcutaneously once a week on day 5 after the estrogen injection.

Six hours after relaxin administration the symphysis of the animal is palpated. The animal is held head down, ventral side away, between the thighs of the seated observer. The sciatic crests and symphysis pubis are firmly grasped between the thumbs and forefingers so that the two halves of the pelvis may be moved back and forth alternately. If the pubic symphysis is rigid at this time, the estrogen and relaxin priming are continued weekly until marked mobility of the symphysis is observed. The increased flexibility is transient, the peak response occurs at 6 h and subsides 12–24 h after injection. Mobility responses are estimated subjectively and scored on an arbitrary scale of 0–6. "Zero" indicates no detectable flexibility of the pubic symphysis, whereas "6" represents extreme softening. Scoring should be performed by the same investigator throughout a study.

One week after an animal has responded positively to 20 µg of relaxin standard, it is added to the assay colony. Before assay time, all eligible animals are mixed and divided into groups of 10–20 each. On the day of the experiment, all animals are palpated before injection. Only those with no symphyseal movement are used. Two doses of test preparation and two doses of standard are injected subcutaneously to different groups. Six hours later, two operators palpate and score each animal.

EVALUATION

The scores are averaged and a median score calculated. The activity of an unknown preparation is determined by comparison with the dose-response curve of concomitantly administered relaxin standard.

MODIFICATIONS OF THE METHOD

Steinetz and Lust (1994) reported that the relaxin-induced pubic symphyseal relaxation in guinea pigs is inhibited by treatment with glycosaminoglycan polysulfates or pentosan polysulfate. The authors recommended the guinea pig symphysis assay for relaxin as a novel rapid screening test for compounds with potential chrondroprotective activity.

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N.10.2.3 Relaxin bioassay in mice

PURPOSE AND RATIONALE

Increase in length of the interpubic ligament in mice can be used as bioassay for relaxin. X-ray measurement of pubic separation (Hall 1948; Dorfman et al. 1953) as well as direct measurement of interpubic ligament (Kroc et al. 1959; Steinetz et al. 1960) have been used.

PROCEDURE

Virgin female mice (e.g., NMRI strain) weighing 18–20 g are used for assay. Twenty mice are employed for each of three dose levels of standard and test preparation. On day zero each mouse is primed with a single subcutaneous injection of 5 µg estradiol cyclopentylpropionate in 0.1 ml sesame oil. On day 7, the test preparation and the standard are injected subcutaneously. At 18 to 24 h later, the mice are sacrificed, the abdominal cavities are opened, and uteri are examined for evidence of estrogen priming. Mice exhibiting threadlike uteri are discarded. The anal and vulval areas are then cut away with scissors, and the upper half of the body is cut off to prevent subsequent bleeding at the pubic symphysis. The bony birth canal is freed of skin, vagina, rectum, and fascia are cleaned off the symphysis pubis. The pelvis is placed on a binocular microscope fitted with a calibrated ocular micrometer. A transilluminating device consisting of a U-shaped lucite rod is affixed to the microscope. The tip of the rod is beveled to direct light vertically through the exposed pubic ligament. The feet of the carcass are grasped between the thumb and index finger, applying a slight lateral traction.

The shortest distance between the edges of the pubes is measured, using the ocular micrometer. Micrometer readings are then converted to millimeters.

EVALUATION

Mean values of length of interpubic ligament, expressed in millimeter, are plotted versus logarithm of dose. From dose-response curves activity ratios with confidence limits versus the standard are calculated.

MODIFICATIONS OF THE METHOD

Bullesbach and Schwabe (1996) tested rat relaxin and synthetic analogs in the mouse symphysis pubis assay for structure-activity relationships.

Samuel et al. (1998) studied the effects of relaxin, pregnancy and parturition on collagen metabolism in the **rat** pubic symphysis. During pregnancy and particularly during birth, there was a significant reduction of tissue wet and dry weight, which coincided with an increase in water content and a significant reduction of overall collagen content.

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N.10.2.4 Inhibition of uterine motility

PURPOSE AND RATIONALE

This is one of the historical assays based on a nonspecific biological response. Its application and reliability depends on the state of purification of relaxin preparations. Relaxin specifically inhibits the spontaneous motility of estrogen-dominated uterus *in vivo* and *in vitro* (Downing and Hollingsworth 1993). Wiqvist and Paul (1958) proposed a relaxin assay based on inhibition of motility of the rat uterus *in vitro*.

PROCEDURE

The donors (female Sprague-Dawley or Wistar rats weighing 150–180 g) are ovarectomized and primed with 2 µg estradiol daily for 3 days. On day 4, uterine horns are removed, bisected, and suspended in Locke's solution gassed with 1% CO_2 in O_2 at a temperature of 37.5 ±0.5 °C. The contractions are recorded using a Statham transducer and a polygraph. A symmetrical 4-point assay is adopted with 2-fold dose-increments of standard and test preparation. Responses consisting of slowing down the contraction frequency up to

total suppression of the contractions are classified visually and assigned score values of 1–3. All four test doses (2 doses each of standard and test preparation) are run simultaneously on the 4 uterine segments obtained from each rat.

EVALUATION

Mean values of scores are calculated for each dose. Potency ratio with confidence limits are calculated from the 2 + 2-point assay.

MODIFICATIONS OF THE METHOD

Felton et al. (1953) described a test for relaxin activity using the inhibition of uterine contraction in anesthetized guinea pigs *in vivo*.

Inhibition of spontaneous and prostaglandin-driven myometrial activity by relaxin in anesthetized rats was reported by Porter et al. (1979).

Downing and Sherwood (1985) studied the influence of relaxin on uterus contractility and on cervical distensibility in different stages of pregnancy in the rat.

Del Angel Meza et al. (1991) measured the effect of relaxin on uterine and ileum tissue of rats *in vivo* and *in vitro*.

Vu et al. (1993) tested the activity of a recombinant prorelaxin in an *in vitro* bioassay in CHO cells. Human uterine endometrial cells were treated with various dilutions of conditioned medium and the amounts of intracellular cAMP produced were determined by radioimmunoassay.

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N.10.2.5
Relaxin assay by interstitial collagenase activity in cultured uterine cervical cells

PURPOSE AND RATIONALE

As an example for the changing approach in assay methods, Mushayandebvu and Rajabi (1995) provided evidence for a biological response suitable for *in vitro* assay. Relaxin is involved in cervical dilatation by stimulating interstitial collagenase, a key enzyme involved in this process. Human recombinant relaxin induces a dose-dependent increase of collagenase activity in cultured guinea pig cervical cells.

PROCEDURE

Abdominal hysterectomy is performed in anesthetized female Hartley guinea pigs under aseptic conditions. The cervix is excised and washed three times in Hank's balanced salt solution (HBSS) containing penicillin G sodium (100 U/ml), streptomycin sulfate 100 µg/ml) and amphotericin B (1.25 µg/ml). The cervix is cut into 2–4 mm pieces and digested in Dulbecco's modified Eagle's medium (DMEM) containing bacterial collagenase type A1 (0.5 mg/ml) and DNase type 1 (0.05 mg/ml) at 37 °C. The digested mixture is filtered once every 15–30 min through a nylon monofilament with a pore size of 400 µm. Following each filtration, separated cells are collected by centrifugation at 365 *g* for 10 min at room temperature followed by a resuspension in DMEM containing 10% heat-inactivated fetal calf serum. Cells are washed twice with HBSS; this is followed by resuspension in DMEM containing 10% penicillin G sodium (100 U/ml), streptomycin sulfate 100 µg/ml) and amphotericin B (1.25 µg/ml). The unfiltered material is redigested and the process is repeated until the cervical tissue is completely digested.

Cells are plated in 24-well plates at 1×10^5 cells per well in 500 µl of medium containing 10% FCS and antibiotics and then are incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C. After 4 days all viable cells are adhered. Culture medium and unattached cells are removed and fresh medium is added. At confluency (5–7 days), culture medium is removed, cells are washed once with HBSS, and serum-free DMEM containing antibiotics is added. The cells are treated with recombinant human relaxin (1–1 000 ng/ml) at 0, 24, and 48 h. Culture media are collected at 96 h from initial treatment with the appropriate controls and frozen immediately at –20 °C until assayed for collagenase activity (Dean and Woessner 1985; Rajabi et al. 1988, 1991).

Enzyme samples (up to 100 µl in assay buffer [50 mM Tris HCl (pH 7.5), 0.2 M NaCl, 10 mM $CaCl_2$, 0.02% sodium azide, 0.035% Brij-35]) are incubated with 10 µl of [^{3}H]telopeptide-free collagen substrate (specific ac-

tivity 3.1×10^6 cpm/mg collagen at 2.24 mg/ml, in 50 mM Tris HCl [pH 7.6], 0.3 M NaCl) in 1.5 ml microfuge tubes. Aminophenylmercuric acetate (0.5 mM) is used was used to activate procollagenase; 1,10-phenanthroline (1 mM) is added to inhibit collagenase and served as blank control for nonspecific collagenolysis. After 18–48 h incubation at 29 °C, the reaction is terminated by addition of EDTA to a final concentration of 40 mM. Collagenase cleavage products are further digested by trypsin and chymotrypsin in the presence of BSA (10 mg/ml assay buffer) for 2 h at 29 °C. The undigested [^{3}H]telopeptide-free substrate is precipitated in 10% trichloroacetic acid at 0 °C for 30 min followed by centrifugation at 18 000 *g* at 4 °C for 30 min. A 100-µl aliquot of the supernatant is mixed in 5 ml Aquasol scintillation fluid and counted in a liquid scintillation counter.

EVALUATION

The percentage digestion is calculated as the total cpm in the supernatant minus cpm in the 1,10-phenanthroline blanks divided by original counts in the [^{3}H]telopeptide-free substrate (in 100 µl of 10% trichloroacetic acid in assay buffer) × 100. One milliunit of collagenase is defined as the amount of collagenase that digests 1 nanogram of collagen in 1 min at 29 °C.

All results are presented as the mean ±SEM of three separate experiments. Statistical analysis is performed by Student's *t*-test for paired observations and by one-way ANOVA for multiple observations.

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N.10.2.6 Relaxin receptor binding

PURPOSE AND RATIONALE

Using a monocomponent, high specific activity, carrier-free porcine relaxin tracer [^{125}I], Yang et al. (1992) demonstrated relaxin receptors in the symphysis pubis, uterus and ovary of mice. A linear Scatchard plot suggested the presence of only one kind of receptor and a dissociation constant of 5×10^{-10} M.

PROCEDURE

Crude membranes are prepared from uterine horns of estrogen-primed mice. The tissues are homogenized three times for 10 s with a Polytron homogenizer. The homogenate is centrifuged at 700 *g* for 10 min, and the supernatants are recovered and recentrifuged twice at the same speed. Thereafter, the supernatants are centrifuged at 10 000 *g* for 30 min. The crude membrane pellets are washed with HEPES buffer, centrifuged again, the resuspended in 200–500 µl water, and aliquots are removed for Lowry protein estimates.

The crude membrane suspension is diluted to 3 mg/ml protein with binding buffer (25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 1.6 mM $CaCl_2$, 0.025 mM $MgCl_2$, 1.5 mM $MnCl_2$, 1%BSA, 2.8 mM glucose, 0.2 mM phenylmethylsulfonylfluoride, 80 mg/l soybean trypsin inhibitor, pH 7.5). The assay is initiated by adding to 120 µl crude membranes (300 µg protein)with and without 0.2 µg unlabeled relaxin. At the end of the incubation period, 1 ml HEPES buffer, containing 1% of BSA, is added, and bound and free relaxin are separated by three successive washings and centrifugations.

EVALUATION

The tubes are analyzed for radioactivity in a Minigamma 400 (LKB, Rockville, MD). Duplicates are run for each experimental point, and experiments are repeated several times.

MODIFICATIONS OF THE METHOD

The control of relaxin secretion and relaxin receptors by relaxin was studied by Bryant-Greenwood et al. (1982).

Relaxin receptors in the myometrium of rats and pigs were studied by Mercado-Simmen et al. (1982a,b).

Fluoresceinylthiocarbamyl relaxin was prepared by Segaloff and Gabbard (1982) for the demonstration of relaxin receptors.

Experiments by Büllesbach and Schwabe (1988) suggested a unique site for the interaction of relaxin with its uterine and symphyseal receptors.

Using a ^{32}P-labeled human relaxin, Osheroff and Phillips (1991) localized relaxin binding sites in rat uterus, cervix and brain.

The receptor binding site of human relaxin II was studied by Büllesbach et al. (1992).

Min and Sherwood (1996) identified specific cell types that contain relaxin receptors in various organs of pregnant pigs.

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N.10.3 Calcitonin gene-related peptide

N.10.3.1 General considerations

In relation to the established traditional hormone definition, many new polypeptides have been identified by molecular biology and characterized by receptor binding and *in vitro* systems, while their definitive function is not yet assigned. Calcitonin-gene-related peptide (CGRP) is an example of this rapid development and change in approach by applying new methods. Calcitonin-gene-related peptide (CGRP) is a 37-amino acid neuropeptide which has been first identified as a product of the calcitonin gene by alternative splicing (Amara et al. 1982). This original peptide is referred to as α-CGRP; a second gene, unrelated to calcitonin, produces β-CGRP (Amara et al. 1985). These two forms are closely related in both rat and man, and are widely distributed throughout most parts of the nervous system. CGRP shares about 50% identity with another 37-amino acid peptide, amylin (Rink et al. 1993). CGRP shows weak but significant homology with adrenomedullin, a 52-amino acid peptide (Kitamura et al. 1993). Several forms of CGRP have been sequenced: α- and β-CGRP from rat and human, and single variants from sheep, pig, chick, salmon, and the laughing frog, *Rana ranri* (Poyer 1997). The calcitonin/CGRP gene is expressed in specific cell types of both the endocrine and nervous systems. The gene is alternatively spliced to yield mRNA encoding calcitonin in thyroid C-cells or the neuropeptide CGRP in a subset of central and peripheral neurons (Amara et al. 1982; Morris et al. 1984; Born and Fischer 1993). The rat as well as the human calcitonin/CGRP gene consists of 6 exons. The calcitonin mRNA contains exons 1 to 4 with a poly(A) tail at exon 4 whereas CGRPα includes exons 1,2,3,5 and 6 with a poly(A) tail at exon 6 (van Rossum et al. 1997).

α- and β-CGRP display several biological activities, including peripheral and cerebral vasodilatation, blood pressure lowering effect, cardiac acceleration, regulation of calcium metabolism, reduction of intestinal motility, regulation of glucose metabolism, stimulation of pancreatic enzyme secretion, diminution of appetite, reduction of growth hormone release, influence on inflammation and nociception, inhibition of interleukin 2 production (Poyner 1992, 1997; Wang et al. 1992; Wimalawansa 1996).

Nuki et al. (1994) compared the vasodilating activity of chicken calcitonin gene-related peptide with human α-CGRP and rat CGRP in the precontracted mesenteric vascular bed of rats (see A.8.2.5).

Wisskirchen et al. (1998) tested agonists of calcitonin gene-related peptide, homologues and antagonists in rat isolated pulmonary artery (see A.1.2.1) and rat deferens (see A.1.2.3).

Tomobe et al. (1998) found that the vasodilation in isolated superior mesenteric arteries by calcitonin gene-related peptide was significantly larger in spontaneously hypertensive rats than in normal Wistar-Kyoto rats.

In order to elucidate the mechanism of endogenous CGRP release in peripheral vasodilation, Brain et al. (1993), used a multiple site ^{133}Xe clearance technique.

Raddino et al. (1997) studied the mechanism of action of human calcitonin gene-related peptide in rabbit heart and human mammary arteries.

Champion et al. (1997) analyzed the responses of human synthetic adrenomedullin and calcitonin gene-related peptides in the hindlimb vascular bed of the cat (see A.8.2.1).

Castellucci et al. (1993) investigated the vasodilator activity of CGRP in the rat isolated and perfused kidney.

McMurdo et al. (1997) investigated the effect of the calcitonin gene-related peptide receptor antagonist $CGRP^{8-37}$ on blood flow in the knee joint of anesthetized rats. Synovial blood flow was measured in both exposed and intact, skin-covered knees by Laser Doppler perfusion imaging.

Sakai et al. (1998) found a synergism of calcitonin gene-related peptide with the blood pressure lowering effect of adenosine.

Preibisz (1993) reported a beneficial effects of CGRP infusions in patients with congestive heart failure and in subjects with neurological deficits after surgical treatment of subarachnoid hemorrhage.

Cadieux et al. (1999) described bronchoprotector properties of calcitonin gene-related peptide in guinea pig and human airways. Calcitonin gene-related peptide inhibited substance P-induced bronchoconstriction *in vivo* and *in vitro*.

Smith et al. (1993) tested the ability of C-terminally truncated fragments of human α-calcitonin gene-related peptide to stimulate amylase secretion from guinea pig pancreatic acini and to relax precontracted mesenteric arteries (see A.1.2.9).

Meini et al. (1995) investigated the propagation of impulses in the guinea-pig ureter and its blockade by calcitonin gene-related peptide. Furthermore, Maggi et al. (1995) studied the mechanisms of the inhibitory effect exerted by calcitonin gene-related peptide on the spontaneous activity of the guinea pig isolated renal pelvis.

Protective effects of calcitonin gene-related peptide in different experimental models of gastric ulcers (reserpine-induced gastric lesions, ethanol-induced gastric lesions, gastric damage and acid secretion in pylorus-ligated rats) were reported by Clementi et al. (1993).

The role of nitric oxide in the anti-ulcer activity of calcitonin gene-related peptide was investigated by Clementi et al. (1994).

Evangelista and Renzi (1997) investigated the protective role of endogenous and exogenous calcitonin gene-related peptide in water immersion stress-induced gastric ulcers in rats.

Li et al. (1997) determined the ability of analogues of human α-calcitonin gene-related peptide to stimulate amylase secretion from guinea pig pancreatic acini (see J.7.0.10) and to relax isolated porcine coronary arteries precontracted with 20 mM KCl (see A.3.1.3).

Calcitonin gene-related peptide acutely augments the contractile response of skeletal muscle to both direct and indirect stimulation as studied in the isolated rat diaphragm by Fleming et al. (1993).

Dumont et al. (1997) used the isolated guinea pig heart (see A.3.1.2) and the isolated rat vas deferens (see A.1.2.3) for *in vitro* bioassays of CGRP agonists and antagonists.

Poyner et al. (1999) found a concentration dependent inhibition of the electrically stimulated twitch response of guinea pig vas deferens by calcitonin gene-rated peptide, amylin and adrenomedullin. (see A.1.2.3).

The role of calcitonin gene-related peptide in protection of capsaicin-induced gastric mucosal hyperemia in rats was studied by Merchant et al. (1994).

Clementi et al. (1994) studied the anti-inflammatory activity of calcitonin gene-related peptide in cutaneous inflammation induced by Croton oil, arachidonic acid, tetradecanolphorbol acetate or cantharidin.

Schaible (1996) investigated the role of tachykinins and calcitonin gene-related peptide in the spinal mechanisms of nociception and in the induction and maintenance of inflammation-evoked hyperexcitability in spinal cord neurons.

The development of tolerance to spinal morphine analgesia in rats was prevented by a calcitonin gene-related peptide receptor antagonist (Menard et al. 1996).

Lutz et al. (1997) investigated the anorectic effects of CGRP and amylin in rats chronically cannulated in the lateral brain ventricle.

Specific calcitonin gene-related peptide receptors were characterized in hamster pancreatic cells (Barakat et al. 1993).

In doses of 25–200 μg/kg i.p., CGRP decreased food intake in mice, suggesting a role for CGRP as a satiety factor (Morley et al. 1996).

CGRP inhibits insulin-stimulated glycogen synthesis in rat skeletal muscle (Leighton and Cooper 1988).

Chatzipantelli et al. (1996) described the lipolytic actions of calcitonin gene-related peptide.

Howitt and Poyner (1997) determined the effects of a series of agonists and antagonists on the calcitonin gene-related peptide receptor of cultured rat L6 skeletal myocytes.

Kurz et al. (1995) studied the receptors of calcitonin gene-related peptide in the rat thymus and suggested that CGRP is a paracrine thymic mediator that may influence the differentiation, maturation and proliferation of thymocytes.

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N.10.3.2 Receptor binding of CGRP

PURPOSE AND RATIONALE

Calcitonin gene-related peptide receptors are localized in many tissues, such as brain, heart, spleen, blood vessels, liver, lung, and kidney (Born and Fischer 1993; Muff et al. 1995). They are abundant in the brain, and the pattern is similar in the rat, pig, cow, sheep and man (Wimalawansa and El-Kholy 1993).

Multiple CGRP receptors (subclasses) have been observed: based on pharmacological properties they are divided into at least two subtypes and denoted as $CGRP_1$ and $CGRP_2$ (Dennis et al. 1989, 1991; Quirion 1992). CGRP-(8-37), which lacks 7 terminal amino acid residues, is a selective antagonist of $CGRP_1$ receptors, whereas the linear analog of CGRP, diacetoamidomethyl cysteine CGRP (Cys[ACM2,7]CGRP), is a selective agonist of $CGRP_2$ receptors.

An atypical CGRP subtype has been reported (Dennis et al. 1991; van Rossum 1997).

A sensitive and specific radioreceptor assay for calcitonin gene-related peptide was described by Wimalawansa (1989).

PROCEDURE

For membrane preparation, tissue samples, e.g., dissected brain areas from rats, are placed in 10 vol of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose, 0.5 mM dithiothreitol and 5 mM EDTA. After centrifugation of the homogenate at 100 *g* for 10 min, the resultant pellet is rehomogenized with 5 vol of fresh buffer and recentrifuged. The pellet is discarded and the supernatants are pooled and recentrifuged at 30 000 *g* for 45 min at 4 °C. The resulting crude membrane pellet is resuspended in 5 vol of assay buffer (50 mM Tris-HCl, 10 mM KCl, 3 mM sodium azide and 200 IU/ml aprotinin) and recentrifuged at 30 000 *g* for a further 45 min. This procedure is repeated twice with fresh assay buffer. Protein concentrations are determined and adjusted to 1–2 mg/ml. The samples are frozen on dry ice and stored at –70 °C until radioligand binding studies.

For radioligand binding studies, [^{125}I]CGRP (25 pM, ~5 fmol containing ~20 000 cpm/tube) is incubated with various membrane preparations (150–200 µg/ml membrane protein in an incubation medium of 200 µl) in the presence of 200 IU/ml aprotinin and 0.2% heat-inactivated BSA at 4 °C for 120 min in polypropylene microcentrifuge tubes in a shaking water bath. At the end of the incubation period, 700 µl of chilled assay buffer are added and immediately centrifuged at 11 000 *g* for 2 min a refrigerated microcentrifuge. The supernatant is discarded and the resulting membrane pellet is rewashed and the radioactivity remaining in the pellet is counted.

EVALUATION

Specific binding is calculated by subtracting the [^{125}I]CGRP binding in the presence of unlabeled CGRP. Results are expressed as fmol of [^{125}I]CGRP bound/mg membrane protein.

MODIFICATIONS OF THE METHOD

Van Rossum et al. (1994) described the binding profile of a selective calcitonin gene-related peptide receptor antagonist ligand, [^{125}I-Tyr]h$CGRP_{8-37}$, in rat brain and peripheral tissues.

Aiyar et al. (1996) described a cDNA encoding the calcitonin gene-related peptide type 1 receptor. Stable expression in human embryonic kidney 293 (HEK 293) cells produced specific, high affinity binding sites for CGRP that displayed pharmacological and functional properties very similar to native human $CGRP_1$ re-ceptor.

Juaneda et al. (2000) reviewed the molecular pharmacology of CGRP and related peptide receptor subtypes.

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N.10.4 Inhibin

N.10.4.1 General considerations

Inhibin is a gonadal dimeric glycoprotein which has an inhibitory effect on the secretion of follicle stimulating hormone by the pituitary gland. The existence of a non-steroidal gonadal hormone was described already by McGullagh in 1932. The hormone was isolated and purified from human seminal plasma, ram rete testis fluid, and from bovine follicular fluid (Franchimont et al. 1979, 1989; Robertson et al. 1986; Vale et al. 1986; de Kretser and Robertson 1989). After the determination of the full structure of bovine (Forage et al. 1986) and human (Mason et al. 1986; Stewart et al. 1986; Tierney et al. 1990) inhibin, it was found that inhibin shares structural homology with a family of glycoproteins which includes Mullarian inhibiting substance, transforming growth factor-β, follistatin, activin and bone morphogenic proteins (Robertson 1991; Moore et al. 1994). Inhibin is a disulfide-linked dimer of an α-subunit and a structurally related β-subunit, either β_A or β_B (Robertson et al. 1992). Inhibin A and inhibin B are related dimeric protein hormones and endocrine regulators of the reproductive axis which show differing pattern during the period of follicular development (Woodruff et al. 1996).

Clinically, inhibin determinations may have uses in the diagnosis of some forms of cancer including granulosa cell tumors, cystadenocarcinoma of the ovary and hydatiform mole and in physiology and pathology of pregnancy including placental function (Halvorson and DeCherney 1996).

An International Standard for porcine inhibin was described by Gaines Das et al. 1992). An International Standard for human recombinant inhibin was established by Rose and Gaines Das (1996).

Tio et al. (1994) purified gonadotropin surge-inhibiting factor (GnSIF), a monomeric polypeptide that shares some biological activities with inhibin, from Sertoli cell-enriched medium.

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N.10.4.2 *In vitro* bioassay for inhibin

PURPOSE AND RATIONALE

Several modifications of *in vitro* bioassays for inhibin were used for establishment of the International Standard for porcine inhibin (Gaines Das et al. 1992) and for human recombinant inhibin (Rose and Gaines Das 1996). Mason et al. (1996) used rat anterior pituitary cells for characterization and determination of the biological activities of noncleavable high molecular weight forms of inhibin A and activin A.

PROCEDURE

Anterior pituitary cells are prepared from adult male rats and added to 48-well cluster plates at an initial density of 75 000 cell per 0.4 ml of medium per well. Cultures are preincubated in DMEM-Ham's F12 (1 : 1) medium with bicarbonate supplemented with nonessential amino acids, antibiotics (100 U/ml penicillin, 100 pg/ml streptomycin, and 250 ng/ml fungizone), and 10% charcoal-stripped FBS. Two days later, the cells are washed, and the medium is replaced with serum-free medium supplemented with antibiotics and recombinant human inhibin A (20 pM–100 pM) as standard. Cultures are incubated with test samples for 65–72 h, after which the samples of medium are collected and stored at –20 °C until assayed for FSH by RIA using goat second antibody precipitation with the following reagents (NIDDK): rat FSH-RP-2, rat FSH-I-8 iodinated using iodogen, and anti-rat FSH-S-11 primary antiserum. Each sample for bioassay is tested at least twice in independent cell cultures to confirm the observations.

EVALUATION

Characteristics of the concentration-response curve, including the maximum effect and medium inhibitory concentration (IC_{50}) are computed using the Allfit program (Munson and Rodbard 1980).

MODIFICATIONS OF THE METHOD

Robertson et al. (1991) studied *in vivo* the FSH-suppressing activity of human recombinant inhibin A in the serum of male and female rats.

Wreford et al. (1994) studied the age-dependence of gonadotropin-suppressing activity of human recombinant inhibin in the serum of male rats.

Simpson et al. (1992) induced bilateral cryptorchism in adult male Sprague Dawley rats under ether anesthesia by cutting the gubernaculum of both testes, translocating the testes to the abdominal cavity and ligating the inguinal canal to prevent redescent of the testes into the scrotum. After 28 days, Sertoli cell cultures from these rats were prepared and inhibin secretion in response to follicle-stimulating hormone was measured.

Brown et al. (1991) investigated the effects of inhibin-rich porcine follicular fluid administration on serum bioactive and immunoreactive FSH concentrations and compensatory testosterone secretion in hemicastrated adult rats

Hertan et al. (1999) used primary cultures of ovine anterior pituitary cells for bioassays of inhibin and identified high affinity binding sites for inhibin using iodinated recombinant human 31-kDa inhibin.

Jakobowiak et al. (1989) found similar effects of inhibin and cycloheximide on gonadotropin release in superfused rat pituitary cell cultures.

Demura et al. (1996) studied the levels of inhibin α, β_A and β_B subunit mRNAs by a quantitative reverse transcription-polymerase chain reaction and the changes in their levels by adding inhibin α, β_A and β_B subunit mRNA antisense oligonucleotides and inhibin A, activin A or GnRH to cultured rat anterior pituitary cells.

Robertson et al. (1996) investigated the specificity of several immunoassay methods in terms of their ability to detect the range of inhibin forms found in plasma and their relationship to bioactivity.

A commercially available enzyme-linked immunosorbent assay (ELISA) for inhibin A (Serotec, Oxford) was used by several authors (Wenstrom et al. 1997; Blumenfeld et al. 1998; Wallace et al. 1998). Magoffin and Jakimiuk (1998) used specific and sensitive two-site enzyme-linked immunosorbent assays for determination of inhibin A, inhibin B and activin A.

An *in vitro* method has been developed by Allenby et al. (1991) for culturing isolated seminiferous tubules from adult rats for 1–3 days and optimized on the basis of the secretion of immunoactive inhibin under basal conditions and after maximal stimulation with rat FSH or dibutyryl cAMP. Inhibin was measured using a double-antibody radioimmunoassay based on an antibody generated in a sheep to the 1–26 sequence (plus gycine^{27}tyrosine^{28}) of the N-terminus of the

α-subunit of porcine 32-kDa inhibin. The effect of three known testicular toxicants (meta-dinitrobenzene, nitrobenzene, and methoxy acetic acid) on these cultures was assessed

Knight et al. (1991) described the development of a two-site immunoradiometric assay for dimeric inhibin using antibodies against chemically synthesized fragments of the α and β subunit, Knight and Muttukrishna (1994) the measurement of dimeric inhibin using a modified two-site immunoradiometric assay specific for oxidized (Met O) inhibin.

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N.10.5 Activin

N.10.5.1 General considerations

Activin is a pluripotent growth factor that was originally isolated based on its ability to stimulate follicle-stimulating hormone, but is now known to have other important roles during development, erythropoesis, inflammation and wound healing (DePaolo 1997). Three types of activin, formed by dimerization of two inhibin-β subunits, β_A and β_B, are termed activin A ($\beta_A\beta_A$), activin AB ($\beta_A\beta_B$) and activin B ($\beta_B\beta_B$) (Ling et al. 1986; Vale et al. 1986; Mason et al. 1989; Nakamura et al. 1992). In addition, a third β-subunit (β_C) has been identified (Hötten et al. 1995; Loveland et al. 1996). Lee et al. (1989) suggested that in the testis, the Leydig cells secrete activin and the Sertoli cells produce inhibin, or a combination of both. As members of the transforming growth factor beta (TGF-β) family, the activins are involved in a diverse range of physiological processes, including the regulation of FSH biosynthesis and secretion (Mason 1988; MacConnel et al. 1999), steps in embryonic development (Thomsen et al. 1990), spermatogonial mitosis (Mather et al. 1990) and erythroid differentiation (Eto et al. 1986). Activin acts via a family of activin receptor subunits that includes one type I (Act RI or ALK-2) and two homologous type II (IIA and IIB) subunits (Dalkin et al. 1996; Hashimoto et al. 1998).

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N.10.5.2 *In vitro* bioassay for activin

PURPOSE AND RATIONALE

In vitro bioassays for activin have been developed using the production of FSH from cultured pituitary cells (Robertson et al. 1992), the accumulation of hemoglobin in K562 erythroleukaemia cells (Schwall and Lai 1991), and the ability to induce mesoderm tissue in animal cap explants of *Xenopus* (de Winter et al. 1992). One of the sites of production of activin A is the bone marrow (Shao et al. 1992; Yamashita et al. 1992; Uchimaru et al. 1995). Brosh et al. (1995) found that the mouse plasmacytoma cell line, MPC-11 (Laskov and Scharff 1970) was exquisitely sensitive to inhibition by activin A without being influenced by a variety of other cytokines and growth factors. On this basis, Phillips et al. (1999) evaluated the MPC-11 cell line as the basis for an *in vitro* bioassay for activin.

PROCEDURE

MPC-11 plasmacytoma cells are cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 24 mM bicarbonate, L-glutamine, non-essential amino acids and Pen-strep in a 95% air/5% CO_2 atmosphere at 37 °C, and passaged at 1×10^6 cells/25 cm^2 flask every second day. For experiments, 100 µl cell suspension are added to 96-well plates at a density of 1 000 viable cells/well in culture medium containing 25 µM β-mercaptoethanol. Activin, other test reagents and sera (100 µl/well) are added to the cultures diluted in phosphate buffer (pH 7.4) containing 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, 0.01% BSA and Pen-strep. Cells are cultured in the presence of test reagents for two days; on the third day 25 µl [^{3}H]thymidine (0.25 µCi/25 µl, 6.7 Ci/mmol) are added to each well and 24 h later the cells harvested and thymidine incorporation assessed. Proliferation of MCP-11 cells, as measured by thymidine incorporation, is inhibited by increasing doses of activin A or activin B, whereas follistatin, inhibin A, LH, or interleukin-1β are ineffective.

EVALUATION

All test preparations, including activin standards and sera, are assayed in quadruplicate, and experiments are repeated at least twice. Relative bioactivity and parallelism are assessed using parallel-line bioassay statistics.

MODIFICATIONS OF THE METHOD

Mesoderm induction assays in *Xenopus* were used by Wuytens et al. (1999). *Xenopus* embryos were obtained by *in vitro* fertilization. They were maintained

in 10% Normal Amphibian Medium and staged according to Nieuwkoop and Faber (1967). Animal pole regions were dissected from mid-blastula (stage 8) embryos and cultured in 75% Normal Amphibian Medium containing 0.1% bovine serum albumin and wild type or mutant activin (2.5 ng/ml). A preliminary assessment of mesoderm induction was based on the elongation of the animal caps. Animal pole regions were then frozen on dry ice, and expression of the mesoderm-specific gene Brachyury (Xbra) was assessed by RNAse protection analysis.

LaPolt et al. (1989) examined the effects of purified porcine activin on inhibin secretion and messenger RNA levels in cultured granulosa cells obtained from immature, estrogen-treated rats. Western blot analyses performed with affinity-purified antisera to inhibin α- and β_A-subunits revealed that treatment with either FSH or activin increased the secretion of inhibin $\alpha\beta$ dimer, with a further increase after co-treatment.

Attardi and Miklos (1990) examined the effect of purified recombinant human activin A on steady state levels of mRNAs for the gonadotropin subunits in pituitary cell cultures prepared from adult male rats.

Carroll et al. (1991) used rat anterior pituitary cells *in vitro* and determined the apparent half-life of FSHb mRNA in the presence and absence of recombinant human activin A after addition of actinomycin D.

Demura et al. (1993) measured follistatin-free activin and inhibin in the culture medium of porcine granulosa cells by a competitive protein binding assay and N-fragment RIA, respectively. Both activin and inhibin were secreted under the control of FSH and LH.

Miyamoto et al. (1999) investigated the effect of activin A on secretion of LH, FSH and prolactin by female cultured rat pituitary cells at the single-cell level by means of the cell immunoblot assay. Anterior pituitary cells were preincubated with or without activin A for 24 h, after which they were monodispersed and immediately used for cell immunoblot assay.

Peng et al. (1999) examined the expression of activin receptor mRNAs in human ovary and placenta. Primers specific for two type I and two type II receptors (ActR-I, Act-RI_B, ActR-II, and Act-RII_B) were used in polymerase chain reaction to amplify cDNAs prepared from granulosa-luteal cells, placental tissues and trophoblast cells.

Liu et al. (1996) measured release of immunoreactive activin A from cultured rat anterior pituitary cells by a specific radioimmunoassay.

Shinati et al. (1991) developed a radioimmunoassay for the measurement of activin A, which is identical to erythroid differentiation factor.

For measurement of activin in biological fluids by radioimmunoassay, McFarlane et al. (1996) added sodium deoxycholate, Tween 20 and sodium dodecyl sulphate as dissociating agents in order to remove the interference of follistatin.

Knight et al. (1996) developed a two-site enzyme immunoassay for the determination of total activin A concentrations in serum and follicular fluid.

Saito et al. (1991) developed an assay method for activin-binding protein, which exploits its high affinity for sulfated polysaccharides, and used this method to investigate the production of activin-binding protein by rat ovarian granulosa cells *in vitro*.

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N.10.6 Follistatin

N.10.6.1 General considerations

Follistatin is a monomeric glycosylated polypeptide chain which was identified from bovine and porcine follicular fluids on the basis of its inhibition of pituitary follicle-stimulating hormone secretion (Robertson et al. 1987; Ueno et al. 1987; Ying et al. 1987; Bohnsack et al. 2000). Follistatin exerts its inhibitory effect on FSH secretion by neutralizing activin activity (Namakura et al. 1990; Shimonaka et al. 1991; de Winter 1996). Follistatin is able to bind and neutralize the actions of many members or the transforming growth factor-β family of proteins and plays a significant role during organogenesis (Patel 1998). There are two main forms of mature mammalian follistatin which occur as a result of alternative modes of precursor mRNA splicing, giving core proteins of 315 amino acids and the carboxy-truncated variant of 288 amino acids. Further variants in the molecular weight of mature follistatin occur as a result of varying degrees of glycosylation Inouye et al. 1991; Sugino et al. 1993).

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N.10.6.2 Immunoassay for follistatin

PURPOSE AND RATIONALE

Evans et al. (1998) developed an ultra-sensitive two-site enzyme immunoassay for human follistatin.

PROCEDURE

Female Balb/c mice are immunized subcutaneously with 20 µg recombinant human follistatin rh-FS288 in an emulsion with complete Freund's adjuvant. The immunization is repeated on two further occasions at monthly intervals in complete Freund's adjuvant, before finally boosting intravenously with rh-FS288 (total 100 µg in saline). The spleen is removed and the splenocytes are fused to Sp2/0 myeloma cells using polyethylene glycol following a standard fusion protocol (Galfre and Milstein 1981). Hybridoma supernatants are screened on a 96-well plate coated with rh-FS288 (0.2 µg/ml in sodium bicarbonate buffer, pH 9.4). Positive clones are expanded and recloned in methyl cellulose. The supernatants from these clones are titrated against rh-FS288 (0.2 µg/ml) under a standard ELISA protocol (Groome et al. 1995). On the basis of these experiments, clones are selected (29/9 and 17/2) and isotoped with a commercial kit. The clones are grown to produce ascitic fluid in pristane-primed BALB/c mice. Purification of IgG is carried out using protein-G affinity chromatography.

Antibody to a selected clone (29/9) diluted in 0.2 M sodium bicarbonate buffer, pH 9.4, is coated by simple absorption onto 96-well ELISA plates overnight at room temperature (10 µg/ml). The following day, the plates ara banged to dryness on paper towelling, then 100 µl dry coat reagent/well are added to the plates. After 1 h incubation at room temperature the plates are banged to dryness and stored in a sealed box.

Both standard (rh-FS288) and samples are diluted in dissociation solution (84 mmol sodium deoxycholate, 3.4% Tween 20, 1% BSA, 5% mouse serum in PBS). Standards are prepared by serially diluting the stock rh-FS288 to give a high standard of 2 500 pg/ml and a low standard of 19.53 pg/ml.

Duplicate 50 µl amounts of standard or test samples are added to wells on the plate, which is then sealed and incubated overnight stationary at room temperature in a sealed moist box. The following day, the plate is washed and to each well is added 50 µl of approximately 1 µg/ml of Fab fragment of clone 17/2, which has previously been coupled to alkaline phosphatase by heterobifunctional chemistry (Ishikawa et al. 1983). This is diluted in Tris conjugate buffer: 1% BSA in 25 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.5% Tween 20. After 2 h incubation in a moist chamber at room temperature, the plate is washed thoroughly, and banged to dryness on paper towelling. Alkaline phosphate substrate (50 µl) is added to each well and the plate incubated for 2 h stationary at room temperature. Amplifier solution (50 µl) is then added to each well and the ensuing chromogenic reaction is stopped by adding 0.4 M HCl (50 µl/well) once color begins to develop in the zero analyte wells and the top standard has an absorbance of approximately 1.8. The well absorbencies are read at 490 nm with a reference wavelength set at 620 nm using a microplate reader.

EVALUATION

To determine whether dose-response relationships of serially diluted standard and test samples are identical (parallel), the slope values (±95% confidence intervals) of log-transformed data for each response curve are compared by linear regression. The curves are deemed to be parallel if the slopes (±95% confidence intervals) are found to overlap.

MODIFICATIONS OF THE METHOD

Nakamura et al. (1992) investigated the effect of follistatin on activin-induced granulosa cell differentiation in freshly harvested granulosa cells from diethylstilbestrol-treated rats. Activin induced a remarkable change in granulosa cellular morphology from elongated fibroblast-like to round cells, which was prevented by follistatin.

Xiao et al. (1992) studied the effects of activin and follistatin on FSH receptors and differentiation of cultured rat granulosa cells *in vitro*.

DePaolo et al. (1991) determined FSH and LH levels by RIA in the serum of ovarectomized rats and compared *in vivo* the follicle-stimulating hormone-suppressing activity of follistatin and inhibin.

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N.10.7 Further peptide hormones discussed in chapters related to the respective indications

Further peptide hormones are discussed in chapters related to the respective indications such as:

Chapter O
Ophthalmologic activity[1]

O.1 Introduction

Ocular pharmacology shares many methods with other indications and treatment of other organs. Therefore, in many instances, the reader has to be referred to other sections, such as:

- neovasculrization of the cornea to Sect. A.8.3.4,
- topical anesthesia of the cornea to Sect. G.1.3.1,
- influence on α- and β-adrenoreceptors in the mouse iris to Sect. A.1.3.24,
- *in vitro* determination of carbonic anhydrase inhibition to Sect. C.1.1.1,
- electroretinogram in diabetic hyperglycemia and galactosemia to Sect. K.8.1.5,
- effect of streptomycin induced cataract to Sect. K.8.1.6,
- effect on galactose feeding-induced cataract to Sect. K.8.1.7,
- effect on naphthalene induced cataract to Sect. K.8.1.8,
- immunological methods to Sect. I.2.2.16.

Nevertheless, ocular pharmacology is a special issue, which justifies a special chapter in this book.

O.2 Intraocular pressure

O.2.1 Acute measurement of intraocular pressure

PURPOSE AND RATIONALE

For evaluation of drugs, intraocular pressure is measured in normal animals or in animals with experimental glaucoma.

PROCEDURE

New Zealand white rabbits, weighing 2.0–2.5 kg, are acclimated to the laboratory environment at least one day prior to experimentation. Intraocular pressure (IOP) measurements consist of topically administering one drop (approximately 50 µl) of a short-acting local anesthetic (Ophthaine 0.5%) directly on the eyes of an unrestrained rabbit. Approximately 40–45 s following local anesthetic administration, an IOP reading for each eye is taken for 10 s and recorded as mm Hg. IOP is determined by applanation pneumotonometry using an Alcon pneumotonograph modified for the rabbit eye (Vareilles et al. 1977).

On the day of the experiment, two baseline IOP measurements are determined and designated as P_1 and P_2, respectively. The P_1 measurement is recorded 30 min prior to drug administration while the P_2 measurement occurs just prior drug or placebo administration. The P_2 measurement is designated as IOP at zero time and is used as the baseline control value for determination of percent reduction in pressure. Thus, a baseline is determined for each rabbit studied.

[1] Review and contributions by W. H. Vogel.

Immediately following the P_2 measurement, the appropriate concentration of drug or vehicle is administered topically to the right eye of 5 rabbits. This is done by placing three successive 20 µl additions directly onto the eye at two- or three-minute intervals. In addition, during drug application, the lower eyelid is held so that excess fluid is collected in the exposed conjunctival sac. Simultaneously, the left eye receives vehicle with the same regimen as the drug-treated right eye. The vehicle (control) treatment is to monitor potential contralateral ocular effects. Following drug or vehicle treatment, IOP measurement are taken at the following time periods: 0.5; 1.0; 1.5; 2.0; 3.0; 4.0; 5.0 and 6.0 h.

EVALUATION

The *IOP* of each rabbit eye for each time period is initially recorded in mm Hg. However, to account for initial *IOP* differences among rabbits, the changes in mm Hg are normalized by converting to percent reduction in outflow pressure (*ROP*).The *ROP* is calculated according to an equation proposed by Mishima (1981):

$$\% \; ROP = (Po - Pe \,/\, Po - Ep) \times 100$$

where *Po* is the *IOP* reading in mm Hg at zero time, *Pe* is the experimental *IOP* reading at the appropriate time following drug or vehicle administration and *Ep* is the episcleral venous pressure taken as a constant of 10 mm Hg. The percent *ROP* for each rabbit at each time period is recorded and grouped to determine a mean percent *ROP* for each time period as well as the duration of action. The overall activity of the drug based on the mean percent *ROP* for five rabbits is categorized as follows:

% reduction in outflow pressure	activity
0 – 10.0	none
10.1 – 20.0	slight
20.1 – 40.0	moderate
>40	marked

MODIFICATIONS OF THE METHOD

Rowland et al. (1981) studied circadian rhythm in intraocular pressure using repeated tonometry in **rabbits**.

Santafé et al. (1997) studied the hypotensive effects of topically applied calcium-antagonists on intraocular pressure in conscious rabbits using a manual applanation tonometer calibrated by direct manometry.

Okada et al. (1995) studied the *IOP* response to different doses of intravitreally injected endothelin-1 in rabbits using a pneumatotonometer.

Drago et al. (1997) studied the effect of repeated administration of combinations of beta-blockers with pilocarpine on *IOP* and heart rate in conscious rabbits.

Chidlow et al. (1999) investigated the effect of topical application of a HT_{1A} receptor agonist on *IOP* in normotensive rabbits using an applanation tonometer.

Goldblum et al. (2000) studied the effect of an acetylcholinesterase inhibitor on *IOP* in normotensive rabbits using a TonoPen XL.

The effect of various carbonic anhydrase inhibitors on *IOP* pressure was measured by several authors, such as Supuran et al. 1999; Briganti et al. (2000).

Burke and Potter (1986) studied the ocular effects of a relatively selective α2 agonist in cats, rabbits and monkeys. Intraocular pressure was measured using an Alcon pneumatonometer, and pupil diameter under constant illumination using a pupillometer. Furthermore, cat nictitating membrane responses were recorded after injections via the lingual artery into the right external artery supplying the nictitating membrane.

Moore et al. (1995) described long-term non-invasive measurement of *IOP* in the **rat** eye. Rats were anesthetized with short-acting isoflurane inhalant and *IOP* was determined by averaging 15 valid individual readings with a TonoPen 2 tonometer.

Krishna et al. (1995) studied the circadian rhythm of *IOP* in Lewis rats using a TonoPen 1 tonometer.

Diurnal variations of *IOP* in the eyes of awake male brown Norway rats were measured by Moore et al. (1996) using a TonoPen XL tonometer.

Cabrera et al. (1999) found no significant difference of *IOP* in four strains of rats using conscious, unsedated animals.

Jia et al. (2000) determined the diurnal *IOP* response of Brown Norway rat eyes after sclerosis of the aqueous humor outflow pathways and its relationship to optic nerve damage. Hypertonic saline was injected into a single episcleral vein and *IOP* measured in both the light and dark phases of the circadian cycle in awake animals.

John et al. (1997) developed a method to measure *IOP* in living **mice** of genetically different mouse strains. Eyes of anesthetized mice were cannulated with a very fine fluid-filled glass micro-needle. The microneedle was connected to a pressure transducer and the pressure signal was analyzed with a computer system.

Miller and Rhaesa (1996) evaluated the effect of a topical α_2-agonist on *IOP*, pupil size and heart rate in normal **cats**.

Bhattacherjee et al. (1999) trained domestic adult cats to accept topical ocular drug administration and pneumotonometry. Cats received either various corticosteroids or vehicle topically to both eyes three times a day for approximately 28 days. The increase of *IOP* was found to depend on the concentration and structure of the corticosteroid.

Gelatt et al. (1997) evaluated the effects of locally applied pilocarpine preparations on *IOP* and pupil size in glaucomatous Beagle **dogs**.

Peterson et al. (1996) compared readings with the Tono-Pen, a handheld applanation tonometer based on the Mackay-Mark principle, with *IOP* measured manometrically after anterior chamber cannulation through the peripheral cornea with a 26-gauge needle connected to a vertically adjustable reservoir and a pressure transducer in cynomolgus **monkeys**.

For *in vitro* determination of carbonic anhydrase inhibition see C.1.1.1.

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O.2.2 Measurement of intraocular pressure by telemetry

PURPOSE AND RATIONALE

McLaren et al. (1996), Pericot et al. (1996), Schnell et al. (1996) published methods to measure intraocular pressure by telemetry in conscious, unrestrained rabbits.

PROCEDURE

Telemetric pressure transducers

The implantable pressure monitors (Model TA11PA-C40; Data Sciences International, St. Paul, MN) are cylindrical, measure 25 mm in length and 15 mm in diameter, and weigh approximately 9 g. They contain a pressure transducer, a short-range amplitude modulation radiotransmitter, and a battery in a biocompatible case. Pressure is conducted to the transducer through a fluid-filled polyurethane catheter that is 15 cm long and 0.7 mm in diameter. The 3-mm distal tip of the catheter couples the transducer to the surrounding fluid; it is modified with a thin flexible wall, and the end is filled with a biocompatible gel. The thin wall conducts transient pressure changes, whereas the gel prevents mixing of the fluid in the catheter with the surrounding biological fluid and conducts slow pressure changes.

The transmitter broadcasts an amplitude modulation signal to two antenna and receiver assemblies mounted inside the animal's cage.

Implant surgery

The transducers are implanted in rabbits weighing 2 to 3.5 kg by using aseptic conditions under anesthesia with intramuscular injections of 50 mg/kg ketamine and 5 mg/kg xylazine. A 4-cm midline incision is made on the dorsal neck and connective tissue and muscle layer over the vertebral column and between the scapulae are blunt dissected to form a pocket to contain the transducer capsule. This area is covered with moist gaze while the eye is prepared to receive the catheter.

The right eye is used in all experiments. Eyelids are retracted with an infant lid speculum, and a 7-0 silk suture is placed through the mid-corneal stroma approximately 3 mm from the superior limbus. The two ends are weighted to maintain downward rotation of the globe. The conjunctiva is opened 2 to 3 mm temporal to the superior rectus muscle and gently dissected free of the sclera back to the supertemporal orbital rim.

A 10-cm, 17-gauge catheter needle is inserted through the conjunctival opening, advanced to the orbital rim, and pushed through the orbital fascia into the subcutaneous space just behind the orbital rim. It is advanced carefully under the skin to the top of the skull and back between the ears until the tip emerges from the opening at the back of the neck. The needle is flushed with sterile saline to remove any tissue or blood it has collected.

The transducer is dipped in an antibiotic solution (1 000 U/ml bacitracin and 10 mg/ml neomycin in Ringer's solution) and is placed in the pocket formed earlier in the neck. The tip of the cannula is then inserted into the bore of the 17-gauge needle and advanced carefully as far as possible. The needle is withdrawn and removed from the conjunctival opening, leaving the transducer catheter along its path. Approximately 2 cm of the catheter is left extending through the conjunctival opening.

The anterior edge of the conjunctival flap is reflected forward, and the sclera over the trabecular meshwork is scraped with a sharp, rounded blade until the trabecular meshwork is visible as a dark streak. A 21-gauge needle is inserted through this thinned tissue into the anterior chamber and withdrawn, and the anterior chamber is refilled through the same opening with Healon. The catheter is grasped with a tube forceps and is inserted through the opening into the anterior chamber. The tip is advanced to approximately mid pupil. Care is taken not to touch the corneal endothelium.

The catheter is secured to the sclera with three 9-0 or 10-0 nylon sutures. Each is tied securely around the catheter three times and its ends passed through the sclera before they are tied together. One 8-0 silk suture is also tied tightly three times around the catheter to promote formation of granulation tissue. The catheter is pulled gently back toward the transducer to remove the excess loop in the orbit.

The capsule is anchored in the pocket on the neck. Again, care is taken, not to nick or make any sharp bends in the catheter. The muscle layer and the skin are closed so that the entire transducer and the catheter assembly are located internally.

For measurement of intraocular pressure lowering drugs, ocular hypertension caused by injection of 5% glucose is used. The rabbits are left undisturbed for 30 min before the beginning of telemetric intraocular pressure measurements. One measurement is recorded every 15 s for 5 s with a sampling rate of 250 Hz. Fifty µl of test solution or saline are instilled into the conjunctival sac of the implanted eye and intraocular pressure is recorded for the next 60 min. A sterile 5% glucose solution (20 ml/kg) is then injected into the marginal vein of the ear. Intraocular pressure is recorded for the next 40 min. All studies are performed using a cross-over protocol. A wash-out period of 5 days is allowed between experiments.

EVALUATION

For statistical analysis, the average values of data collected over the time period of interest are calculated for individual animals. For comparisons within groups, statistical analysis is performed on averaged date using two-tailed Student's *t*-test for paired data or unpaired test for unpaired data.

MODIFICATIONS OF THE METHOD

Pericot et al. (1996) induced chronic ocular hypertension by a single injection of α-chymotrypsin into the posterior ocular chamber of rabbits anesthetized by an intramuscular injection of 35 mg/kg ketamine and 4 mg/kg xylazine. Continuous intraocular pressure was measured by telemetry and the effects of timolol, dorzolamide, and epinephrine. The transmitter of a miniaturized radiotelemetry system was implanted into rabbits, and its catheter was tunneled subcutaneously to the superior conjunctival sac and inserted into the midvitreous.

Dinslage et al. (1998) recorded the effects of tonometry, handling, water drinking, and instillation of topical ophthalmic medications on *IOP* by telemetry in rabbits for several months during each 24-h day/night cycle.

McLaren et al. (1999) measured the effects of topical ibopramine and epinephrine on the circadian rhythm of *IOP* in undisturbed rabbits by telemetry.

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O.3
Aqueous humor flow rate

PURPOSE AND RATIONALE

Aqueous humor flow rate can be determined by ocular fluorophotometry (Ogidigben et al. 1993, 1994).

PROCEDURE

New Zealand White rabbits weighing 2.5–4 kg of either sex are used. For unilateral sympathectomy, the animals are anesthetized with a dose of ketamine/xylazine (44/8 mg/kg i.v.). Through a midline incision in the neck, the cervical sympathetic nerve trunk is isolated from both the vagus and the common carotid artery by blunt dissection. To be sure that the isolated nerve is the sympathetic trunk, it is electrically stimulated with a square-wave pulse of 4 Hz (0.7-ms duration, 5-s train) at 4 V with a bipolar electrode connected to a Grass SD9 stimulator; dilatation of the rabbit's pupil is satisfactory confirmation of nerve isolation. The preganglionic sympathetic trunk and superior cervical ganglion are severed and removed. During 1–2 weeks post surgery, sympathetically denervated eyes of rabbits are examined for persistent miosis and ptosis.

The corneas of adult rabbits are treated bilaterally by instilling 2% fluorescein topically, 1 drop every 5 min for a period of 30 min (Brubaker 1989). Subsequently, fluorescein concentrations in the cornea and anterior chamber are measured using a Fluorotron Master (Coherent Medical Inc., Palo Alto, CA). Basal aqueous humor flow rates are established both in sympathetically denervated and normal eyes. In the following week, groups of rabbits are treated bilaterally with the test drug or with vehicle. Fluorometry recordings are started 30 min after drug or vehicle administration, and subsequent recordings are made every hour for a total of 5 h.

EVALUATION

Student's *t*-tests for paired data are used to evaluate drug-induced changes.

CRITICAL ASSESSMENT OF THE METHOD

The study in hemi-sympathectomized animals allows the separation of direct drug effects from effects exerted via the sympathetic nervous system

MODIFICATIONS OF THE METHOD

According to Yablonski et al. (1978), the acute decrease in intraocular pressure caused by timolol can be explained entirely by its effect on the aqueous humor flow.

The effect of various compounds on aqueous humor inflow into the posterior chamber of the **rabbit** eye can be used to determine the mechanism of action and to compare the potency of *IOP* lowering compounds. Aqueous humor production is indirectly quantified in the New Zealand white rabbit by means of the *IOP* recovery rate assay of Vareilles and Lotti (1981). After a control pneumotonographic *IOP* measurement, a 20% NaCl solution is infused i.v. into the marginal ear vein of a rabbit at a rate of 1 ml/min for 10 min using an infusion pump. The hypertonic saline infusion causes a rapid and marked fall in *IOP* with a peak reduction occurring 15 min after the start of infusion. Thereafter, the pressure gradually returns to normal over the ensuing 120 min in normal rabbits. Compounds under study are solubilized in a suitable vehicle. Sixty min prior to NaCl infusion, the drugs are instilled directly onto both eyes as three successive 20 µl drops with each drop separated by two or three min. Following the NaCl infusion, the intraocular pressures of both eyes are measured at 5, 10, 15, 30, 60, 90 and 120 min and then every 30 min until *IOP* recovers to preperfusion values. The mean of the *IOP* for the 2 eyes is recorded and plotted against time. The *IOP* recovery rate is then expressed as the slope of the *IOP* recovery line calculated from the points 15 min from the start of NaCl infusion to the ensuing 120 min by means of the least squares method. The difference in slope and percentage change between control and that produced by experimental compounds are analyzed using an unpaired Student's *t*-test. A compound which lowers *IOP* by reducing aqueous humor flow will exhibit a prolonged *IOP* recovery rate as evidenced by a shallow slope compared to that produced by vehicle.

Taarnhøj et al. (1990) reported calibration of measurements *in vivo* of fluorescein in the rabbit cornea. The quenching was measured by four different techniques: (1) by elution of fluorescein, (2) by elution of albumin, (3) by polarization of fluorescence, and (4) by

spectrofluorometry. Quenching of fluorescence of fluorescein in the rabbit cornea can be explained by the interaction of fluorescein and albumin in the cornea.

Burke and Schwartz (1996) surveyed the preclinical data on aqueous humor flow and intraocular pressure of brimonidine.

Beilin et al. (2000) characterized the *IOP* lowering activity and possible mechanism of action of a synthetic, non-psychotropic cannabinoid after i.v. administration in rabbits measuring *IOP* by pneumatonometry, aqueous humor inflow rate by fluorophotometry, blood pressure and heart rate by a computerized physiograph system connected to the central ear artery.

Mermoud et al. (1996) established methods to determine normal outflow facility using anterior chamber infusion with constant pressure and aqueous humor production by a technique of dilution with FITC-albumin in Lewis **rats**.

Tian et al. (1997) determined the effects of adenosine agonists on intraocular pressure with an minified Goldmann applanation tonometer and aqueous humor dynamics by fluorophotometry in cynomolgus **monkeys**.

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O.4 Experimental glaucoma

O.4.1 Alpha-chymotrypsin induced glaucoma

PURPOSE AND RATIONALE

Sears and Sears (1974) induced glaucoma in rabbits by intraocular injection of alpha-chymotrypsin. Besides this, relatively few animal models resembling glaucoma in human beings have been described (Gelatt 1977).

PROCEDURE

Male New Zealand rabbits weighing about 2 kg are pretreated with 10 mg/kg i.p. indomethacin to prevent the otherwise immediate onset of inflammation and then slightly anesthetized with pentobarbital to eliminate any nystagmus. The right eye is anesthetized topically with 2% lidocaine. The anterior chamber is cannulated with a 30-gauge needle attached to a reservoir set at a pressure of 25 mm Hg. Then a second cannula, 32-gauge, is introduced into the anterior chamber near the limbus and directed to the posterior chamber through the pupil. A sterile isotonic saline solution (0.5 ml) containing 150 units of alpha-chymotrypsin is irrigated through the cannula into the posterior chamber. Care is taken to avoid the injection of any enzyme into the corneal stroma. Both cannulae are then removed without significant loss of aqueous humor. The eyes are examined at daily intervals for the first week, then on alternate days for the second week, and then weekly for the duration of the experiments. Intraocular pressure is measured with a tonometer adapted for rabbit eyes.

Treatment with drugs is performed before and after surgery.

EVALUATION

Intraocular pressure of animals treated with alpha-chymotrypsin and those treated additionally with drugs is expressed as mean ±SEM and compared statistically with untreated controls using ANOVA followed by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Melena et al. (1998a) measured the effect of topical dihydroergocristine on *IOP* in alpha-chymotrypsin-induced ocular hypertensive **rabbits** with a pneumatonometer.

Drago et al. (1997) measured the effects of beta-blockers combined with pilocarpine on rabbit intraocular pressure. Experimental increase of intraocular pressure was induced by a subconjunctival injection of betamethasone-21-phosphate (4 mg/ml) in both eyes

every week for 4 weeks. The intraocular pressure was measured in conscious rabbits using an electronic pneumotonometer after surface anesthesia with one drop of 0.4% benoxinate solution.

Melena et al. (1997) induced ocular hypertension in rabbits by weekly subconjunctival injection of a betamethasone suspension into the left eye and measured *IOP* with a manometrically calibrated pneumatonograph.

With the same method, Melena et al. (1998b) measured the effect of topical diltiazem on the rise of *IOP* and the effect on established ocular hypertension.

Santafe et al. (1999) induced ocular hypertension in rabbits by oral administration of 60 ml/kg tap water and measured the effect of topical diltiazem on *IOP*.

Balaban et al. (1997) studied the mechanisms of vasopressin effects on intraocular pressure in anesthetized **rats** after cannulation of the anterior chamber of the eye and infusions of artificial cerebrospinal fluid or arginine vasopressin solutions.

Mermoud et al. (1994) described an animal model of uveitic glaucoma. Uveitis was induced in Lewis rats by injection of S-antigen.

Morrison et al. (1998) produced scarring of the aqueous humor outflow pathways by unilateral episcleral vein injections of hypertonic saline in Brown Norway rats and measured *IOP* and optical nerve damage after topical treatment with artificial tears, 0.5% betaxolol, or 0.5% apraclonidine.

Ueda et al. (1998) described an experimental glaucoma model in the rat induced by laser trabecular photocoagulation after an intracameral injection of India ink.

Gu et al. (2000) induced experimental glaucoma in the right eyes of Wistar albino rats by intracameral injection of India ink followed by laser trabecular photocoagulation 4 days later. The left eye served as control. Drugs were injected intraperitoneally just before trabecular photocoagulation. Five days later, 3% fast blue was injected into both superior colliculi. The eyes were enucleated another 3 days later and flat mounts of the retinas were prepared. Labeled ganglion cells were counted in the area 1 mm away from the optical disc.

Wang et al. (1997) induced glaucoma in one eye of cynomolgus **monkeys** by repeated argon laser photocoagulation or diode photocoagulation of the midtrabecular meshwork to study the effects of 5-methylurapidil.

Schmidt et al. (1998) induced glaucoma in rhesus monkeys by 2–3 laser treatments of one eye and measured *IOP*, ocular pulse amplitude and peak pulse blood volume after treatment with anti-glaucoma drugs.

Serle et al. (1998) studied the effect of prostanoid derivates on *IOP* and aqueous humor flow rates in cynomolgus monkeys with unilateral laser-induced glaucoma.

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O.5 Local anesthesia of the cornea

PURPOSE AND RATIONALE

Surface anesthesia of the cornea is a prerequisite for ophthalmic surgery. Sigmund Freund studied the pharmacological actions of cocaine and Carl Koller introduced cocaine in 1884 into clinical practice as a topical anesthetic. In pharmacology, following the pioneering work of Sollmann (1918), block of the rabbit corneal reflex as described by Régnier (1923) has become a standard test method for evaluating local anesthetics (Quevauviller 1971; Muschaweck et al. 1986). A detailed description of methods to evaluate the activity of compounds for surface anesthesia is given in Sect. G.1.3.1.

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O.6 Experimental cataract formation

PURPOSE AND RATIONALE

Cataract formation can be induced experimentally by various procedures, such as

- **streptomycin** treatment (see Sect. K.8.1.6),
- **galactose feeding** (see Sect. K.8.1.7),
- **naphthalene** treatment (see Sect. K.8.1.8).

Another procedure to induce experimental cataract is treatment of young rats with **selenite**. A review of this model was given by Shearer et al. (1997). Oštádalová et al. (1978) induced cataract by administration of a single dose of sodium selenite to suckling rats.

PROCEDURE

Male Wistar rats are used. On the 2nd day of life, the sucklings are divided in such a way that 8 males were kept with one mother. On the 10th day of life (i.e., when they are in absolute nutritive dependence of the mother) a single dose of 0.02 M solution of Na_2SeO_3 is administered s.c. to 5 experimental groups receiving 5, 10, 20, 40 and 60 µmoles/kg, respectively. The first control group receives s.c. a 0.02 M solution of a sulphur compound (Na_2SO_3), i.e., an element homologous with selenium, at a dose of 60 µmoles/kg. As reference group, 2-months-old male rats are used to which a 0.02 M solution of Na_2SeO_3 is administered s.c. in an amount of 20 µmoles/kg. Mothers of the sucklings as well as the group of adult males, are fed on a standard laboratory diet with water ad libitum. All animals are checked daily and the experiment is terminated 20 days after the treatment.

Cataracts in the sucklings are visible by the naked eye after the opening of their eyes, i.e., on the 14–16th day of life. The opacity is yellowish-white, localized in the center of the lens (nuclear cataract). Two forms are observed: permanent cataracts, persisting through the whole experimental period, and intermittent cataracts, which disappear after some time and reappear after a certain delay. Doses of 40 and 20 µmoles/kg are mostly associated with permanent cataract, the dose of 10 µM/kg causes an uneven distribution of both types of cataracts. The cataractogenic effect is not found in adult animals.

EVALUATION

Dose-dependence of the incidence of cataracts is evaluated using the χ^2-test.

MODIFICATIONS OF THE METHOD

Anderson et al. (1988) observed massive cortical cataract 15–30 days after a single injection of an overdose of sodium selenite into 14-day-old rats.

Watanabe and Shearer (1989) studied the release of crystallins from the lens into aqueous an vitreous humor in rats with a selenite overdose cataract.

Huang et al. (1991) studied DNA damage, repair, and replication in selenite-induced cataract in rat lens.

Huang et al. (1992) observed cataract formation in lenses from rat receiving multiple, low doses of sodium selenite.

Kelley et al. (1993) demonstrated a marked reduction in alpha-crystallin chaperone activity by calpain II in selenite cataract induced in young rats.

Zhao and Shichi (1998) tested the prevention of cataract and other ocular tissue damage induced by injection of 350 mg/kg **acetaminophen** and induction of cytochrome P450 in C57BL/6 mice.

Kojima et al. (1996), Shui et al. (1997) studied cataract formation in Brown Norway rats treated with X-ray irradiation and **corticosteroids** using slit lamp microscopy, photography with a Scheimpflug camera and scanning electron microscopy.

Hatano and Kojima (1996) induced cataract in Brown Norway rats by **UV-B irradiation** and pre-treatment with **l-buthionine sulfoximine**.

Ishida et al. (1997) treated adult rats with oral doses of 0.2, 1 or 5 mg/kf **FK506 (tacrolimus)** over 13 weeks. In the highest dose, cataract development was observed.

Cataract formation in **transgenic mice** was studied by Mitton et al. (1996) and by Cammarata et al. (1999).

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O.7
Models of eye inflammation

O.7.1
Allergic conjunctivitis

PURPOSE AND RATIONALE

Allergic conjunctivitis is a common clinical condition that resembles immediate hypersensitivity reactions in the nose, airways, and skin in many aspects. Allergic conjunctivitis is regarded as a mast cell-mediated disease: a similar array of inflammatory mediators, mostly histamine, is released, and the conjunctiva is responsive to many of these inflammatory mediators.

Trocme et al. (1986), Fukushima et al. (1997), Iwamoto et al. (1999) induced allergic blepharoconjunctivitis in rats by immunization with ovalbumin. Keane-Myers et al. (1999) sensitized and challenged mice by local application of a cat dander extract. Ragweed pollen was used for sensitization of mice by Merayo-Lloves et al. (1996), Hu et al. (1998), Magone et al. 1998, 2000).

PROCEDURE

An extract of ragweed pollen is prepared. Ragweed is defatted with acetone and extracted with 50 mM Tris buffer overnight. The extract is then dialyzed against PBS for 48 h, filtered, and stored at 4 °C until use. Protein concentration is determined with Coomassie plus protein assay. The ragweed extract is biotinylated. An $Al(OH)_3$ gel (alum) is prepared, centrifuged for 15 min at 1 000 rpm and stored at 4 °C. Ragweed and alum are mixed at a concentration of 50 µg ragweed in 5 mg of alum 40 min before immunization. On day 0 mice are anesthetized with methoxyflurane during the injection of ragweed and alum in one hind footpad. On day 10, conjunctivitis is induced by topical application of 1.5 mg ragweed suspended in 10 ml phosphate buffered saline, pH 7.2, into each eye. Two groups of control mice are either not immunized or immunized with alum alone; both groups are challenged with the same dose of ragweed on day 10. The animals are sacrificed 20 min and 3, 6, 12, 24, 48, and 72 h after topical ragweed administration.

Animals are examined clinically for signs of immediate hypersensitivity response 20 min after the topical application of ragweed. Chemosis, conjunctival redness, lid edema, and tearing were each graded on a 0–3+ scale. The total score consists of the sum of scores in each of these four categories.

For histology, the eyes are enucleated with the attached lids and intact conjunctiva and immediately fixed in 4% glutaraldehyde for 30 min and then trans-

ferred to 4% formaldehyde for at least 24 h before processing. The tissue is embedded in methyl acrylate, serially sectioned and stained. The numbers of infiltrating neutrophils, eosinophils, goblet cells, and mast cells in one section are counted. Immunohistochemical staining is performed with rat primary antibodies using an avidin-biotin-peroxidase complex technique. Macrophages and lymphocytes in one section of the conjunctiva are assessed.

EVALUATION

All results are presented as mean ±SE. Comparisons between immunized mice, drug treated mice and controls are made with the two-sided Student's *t*-test.

MODIFICATIONS OF THE METHOD

Hu S et al. (1998) compared effectiveness and molecular pharmacological mechanisms of antiallergic agents on experimental conjunctivitis induced by ragweed in SWR/J mice. Allergic conjunctivitis was evaluated by scoring of the clinical signs and histopathology. mRNA expression of interleukin 1β, IL-6 and tumor necrosis factor α was analyzed by reverse transcription polymerase chain reaction techniques.

Woodward et al. (1995) studied the pruritogenic and inflammatory effects of prostanoids in the conjunctiva in **guinea pigs** and counted the scratching episodes by the hind limb.

Reichel et al. (1998) established a simple model of **conjunctival wound healing** in the mouse eye. Four week old BABL/c mouse eyes were studied over a 14 day period. Surgical procedure under general anesthesia involved a blunt dissection of the conjunctiva performed by injection of 25 µl of phosphate buffered saline via a 27 gauge needle into one eye, while the was used as control.

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O.7.2 Corneal inflammation

PURPOSE AND RATIONALE

Unlike the conjunctiva, the cornea is an avascular tissue. The cornea serves three major functions: protection, refraction and light transmission. Corneal inflammatory diseases usually result from injury and infection rather than from allergic disorders. Models involving chemical or mechanical injury, therefore, directly mimic clinically encountered situations.

Leibowitz and Kupferman (1974) induced inflammatory response by the injection of clove oil directly into the corneal stroma, resulting in the chemotactic attraction of large numbers of polymorphonuclear leukocytes.

PROCEDURE

New Zealand albino rabbits of either sex are anesthetized with 15 mg/kg i.v. sodium thiamylal. A corneal

inflammatory response is produced in both eyes by the interlaminar inoculation 0.03 ml of laboratory grade clove oil centrally with a 30-gauge needle attached to a tuberculin syringe. All animals receive two intravenous injections of 0.05 mCi/kg of tritiated thymidine (0.7 Ci/mM) 24 h apart. The second thymidine injection is given 24 h prior to the induction of the corneal inflammatory response. Therapy is initiated immediately after the intracorneal injection of clove oil consisting of one drop (0.05 ml) of test compound or standard every hour for a total of 6 doses. One h later, the animals are sacrificed under anesthesia and a 10 mm penetrating corneal button is removed by trephination. The tissue samples are solubilized with a commercially available solubilizing agent (Soluene, Packard Instruments Corp.). The samples are counted in a scintillation counter for 10 min and the amount of radioactivity in each cornea documented.

EVALUATION

The data from individually treated eyes are expressed as percent change in radioactivity relative to their own untreated control eyes. These differences are averaged to determine a mean value for each treatment group. Statistical comparisons are performed by standard methods including random block analysis.

MODIFICATIONS OF THE METHOD

Neovascularization of the rabbit cornea has been used by several authors to study inhibition of angiogenesis (see Sect. A.8.3.4).

Alio et al. (1994, 1995) studied the relative effectiveness of nonsteroidal anti-inflammatory drugs versus corticosteroids and of antioxidants in the topical treatment of experimental acute corneal inflammation induced by corneal alkali burn in rabbits. The corneal inflammatory response was evaluated by Luminol-enhanced chemiluminescence, ultrasonic pachymetry, and computer-assisted analysis of the area of corneal ulceration.

Laria et al. (1997) evaluated combined non-steroidal therapy in experimental corneal injury. A cornmeal alkali burn was induced in New Zealand white rabbits by applying 1-N NaOH filter paper on the central axis of the right cornea for 30 s. The inflammatory index, area and perimeter of the wounded corneal zone, and corneal transparency were evaluated after 5 days treatment.

A similar method was used by Ozturk et al. (2000).

Sonoda et al. (1998) studied inhibition of corneal inflammation by the topical use of Ras farnesyltransferase inhibitors. The central corneas of BALB/c **mice** were cauterized with silver nitrate. Clinical signs and neovascularization were evaluated.

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O.7.3 Auto-immune uveitis in rats

PURPOSE AND RATIONALE

The mechanisms of certain types of uveitis have been studied in animals involving autoimmunity. Uveitis has been produced in guinea pigs following injections with homologous uveal tissue.

PROCEDURE

Hartley strain guinea pigs, weighing 400–500 g, are injected at 0, 1, 2, 5, and 8 weeks with a 10% suspension of homologous retina emulsified in Freund's complete adjuvant. The retinas are obtained by dissection of freshly removed eyes of either normal albino or pigmented guinea pigs and stored frozen at –50 °C. Each animal receives 0.2 ml of this emulsion at a time, the first injection being administered intradermally into both hind foot-paws, and the remainder of the series given intramuscularly. Groups of 6 animals receive the test drug or the vehicle orally. Control animals receive similar injections of saline. The severity of the uveal inflammation can be clinically evaluated by slit lamp examination. The animals are sacrificed and the eyes are examined histologically.

EVALUATION

The incidence of uveitis is recorded by histological means as percentage of treated animals.

MODIFICATIONS OF THE METHOD

Highly purified bovine retinal S antigen in complete Freund's adjuvant was injected subcutaneously in **guinea pigs** to induce experimental autoimmune uveitis (Liversidge et al. 1987; Mahlberg et al. 1987).

Cultured retinal pigment epithelial cells from guinea pigs were used by Liversidge et al. (1988).

Experimental autoimmune uveitis has been induced in **rats** (Nussenblatt et al. 1981; Chan et al. 1984, 1987; Mochizuki et al. 1985; Fujino et al. 1988).

Experimental autoimmune uveoretinitis was induced in Lewis rats by injection of very high doses of bovine opsin (Brockhuyse et al. 1987).

Melanin-associated antigen from the iris and ciliar body was used to induce experimental autoimmune anterior uveitis in male Lewis rats by Bora et al. (1995).

Ramanathan et al. (1996) found that recombinant IL-4 aggravates experimental autoimmune uveoretinitis in Lewis rats.

Hanashiro et al. (1997) injected various doses of either lipopolysaccharide or the lipid A region of the lipopolysaccharide into the foot pad of an inbred strain of Lewis rats and compared the inflammation patterns by assessing the protein concentrations and the inflammatory cell content in the aqueous humor.

Merino et al. (1998) reported that lipoteichoic acid from *Staphylococcus aureus* injected into the foot pad of Lewis rats induced a strong ocular inflammation between 24 and 30 h after injection.

Hikita et al. (1995) evaluated the effects of topical FK506 on endotoxin-induced uveitis in the Lewis rat.

Tsuji et al. (1997) investigated the effects of systemically and topically applied betamethasone derivatives on endotoxin-induced uveitis in female Lewis rats.

McMenamin and Crewe (1995) studied kinetics and phenotype of the inflammatory cell infiltrate and the response of the resident tissue macrophages and dendritic cells in the iris and ciliary body in endotoxin-induced uveitis in Lewis rats.

Uchio et al. (1997) achieved suppression of actively induced experimental autoimmune uveoretinitis in rats by $CD4^+$ T cells.

Egwuagu et al. (1999) found in transgenic Lewis rats that expression of interferon-gamma in the lens exacerbates anterior uveitis and induces degenerative changes in the retina.

Immunogenic uveitis in **rabbits** was studied by Kaswan and Kaplan (1988).

Jamieson et al. (1989) sensitized rabbits to bovine serum proteins and then challenged intravitreally to induce an uveitis. They recommended this procedure as a model for anti-inflammatory drug screening.

Whitcup et al. (1996) produced endotoxin-induced uveitis in **mice** by injecting 200 µg *Salmonella typhimurium* endotoxin diluted in 0.1 ml sterile saline into one hindpad.

Geiger et al. (1994) reported that transgenic mice expressing IFN-gamma in the retina develop inflammation in the eye and photoreceptor loss.

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O.7.4 Ocular inflammation induced by paracentesis

PURPOSE AND RATIONALE

Paracentesis stimulates prostaglandin PGE_2 and $PGF_{2\alpha}$ release into the anterior chamber of the eye which can be inhibited by antiinflammatory agents (Miller et al. 1973; Kulkarni and Srinivasan 1985).

PROCEDURE

New Zealand white rabbits (2.0 to 2.5 kg) are anesthetized with a 50 : 50 mixture of xylazine (20 mg/kg) and ketamine (100 mg/kg) i.m. Test compounds are topically administered by placing three drops (about 60 µl) on each eye. The eyes are then taped shut to prevent drying. One hour after compound or vehicle administration, inflammation is induced by paracentesis. For each rabbit, the aqueous humor from both eyes is combined to form the "Pre" sample. Three more drops of compound or vehicle are again administered to each eye and the eyes taped shut. Another one-hour period is allowed to elapse, enough time for the aqueous humor to build back up in the eye. Paracentesis is performed a second time and the aqueous humor from both eyes is combined to form the "Post" sample. Prior to this second paracentesis, the general appearance of the eye is rated on a scale of 1 to 3, based on the following criteria: 1 = clear, relatively normal appearance; 2 = opaque, some protein accumulation in the anterior chamber; 3 = very cloudy, protein accumulates in clumps in the anterior chamber, hyperemia, swelling. This rating gives some indication of the extent of inflammation and correlates with the amount of prostaglandin accumulation. Prostaglandins are measured in 10 µl of aqueous humor from the "Pre" and "Post" samples by RIA.

EVALUATION

The amount of prostaglandin present is determined according to the standard curve supplied with each RIA kit. The extent of inflammation is determined by the magnitude of increase in prostaglandin levels in the "Post" versus the "Pre" sample. Dose-response curves for inhibition by test drug and standard are established.

MODIFICATIONS OF THE METHOD

Struck et al. (1995) studied the effects of non-steroidal anti-inflammatory drugs on inflammatory reactions. The right eye of rabbits was burned with alkali (0.25 M sodium hydroxide). The animal were treated with eye drops containing a cyclooxygenase inhibitor and a leukotriene antagonist. Hyperemia of the limbal vessels, corneal vascularization, the number of PMNLs in the cornea and the prostacyclin level in the anterior chamber of the eye served as criteria.

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O.7.5 Ocular inflammation by lens proteins

PURPOSE AND RATIONALE

Ocular inflammation can be induced by lens proteins which can be prevented by lipoxygenase inhibitors (Miyano and Chiou 1984; Chiou and Chiou 1985; Chang and Chiou 1989).

PROCEDURE

New Zealand albino rabbits are anesthetized by i.m. injection of 25 mg/kg ketamine hydrochloride and 5 mg/kg xylazine. The anesthesia is maintained by injection of 12.25 mg/kg ketamine and 2.5 mg/kg xylazine every hour throughout the experiment. After the animals are deeply anesthetized, 50 µl of the drug is topically applied to one eye and the other eye receives 50 µl of the solvent. One hour after the application of the drug, 25 µl of lens protein (30 mg/ml, prepared according to Miyano and Chiou 1984) is injected intracamerally with a 27 1/2 gauge needle; extreme care is taken to avoid contact with the iris. Fifteen min after injection of lens proteins, 14 mg/kg of fluorescein is infused through the marginal ear vein at a rate of 1 ml/min. The inflammation which occurs in the eyes of the rabbits is measured by quantifying the amount of fluorescein leakage into the anterior chamber using a fluorophotometer. Measurements are taken at various intervals after injection of lens proteins. At the end of experiments, the animals are sacrificed by an overdose of pentobarbital sodium. Fluorescence measurements are done at 30, 60, 90, 120, 180, 240 and 300 min intervals.

EVALUATION

The concentration of fluorescein in the anterior chamber is determined by taking the average of three values in each of the fluorometric measurements. The results are analyzed by using a paired *t*-test with *P*-values af less than 0.05 as statistically significant.

MODIFICATIONS OF THE METHOD

Augustin et al. (1996) evaluated the effects of allopurinol in lens induced uveitis by morphological methods and compared these effects with those of steroids and a combination of both drugs biochemically and morphologically.

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O.7.6 Proliferative vitreoretinopathy in rabbits

PURPOSE AND RATIONALE

Injection of cultured fibroblast cells into the rabbit vitreous results in a condition that mimics the features of proliferative vitreoretinopathy in man (Algvere and Kock 1976; Sugita et al. 1980; Ophir 1982). Proliferative vitreoretinopathy is a complication of severe eye injury or retinal detachment and is a major cause of failure in retinal reattachment surgery. Because of its simplicity and reproducibility, Wiedemann et al. (1984) proposed this model in rabbits as screening model for antiproliferative drugs.

PROCEDURE

Dermal fibroblasts are obtained from skin explants of young rabbits which are prepared by excising a 1×1 cm area of dorsal skin and scraping it thoroughly with a scalpel blade to remove hair and subcutaneous fat. The explants are rinsed in sterile PBS, minced finely, and placed in 35-mm Petri dishes containing 200 µl of Dulbecco's medium supplemented with 20% heat-inactivated fetal calf serum, 50 µg/ml garamycin, and 5 µg/ml fungizone. When cells begin to grow, tissue pieces and medium are removed and 2 ml of fresh medium is added. When cells have grown to confluence, they are passaged into 75-cc flasks by rinsing with 1 ml of PBS and incubating with 1 ml of 0.25% trypsin/0.02% EDTA for 5–10 min at room temperature. Subsequent to the initial passage, cells are split at a 1 : 4 ratio upon reaching confluence. For injection into the vitreous, confluent cells from the third to tenth passage are trypsinized with 0.25% trypsin/0.02% EDTA, centrifuged at 500–600 *g*, washed twice with the medium, then re-suspended in the medium and allowed to stand for 10–15 min to let cell clumps settle. The number of cells in suspension is determined using a Coulter counter. Tuberculin syringes are filled with 600 µl of cell suspension, which allows five injections in 0.01 ml steps.

Pigmented rabbits of either sex weighing 2–3 kg are anesthetized with 2.0–2.5 ml of a 1 : 1 Rompun-ketamine mixture. The eyes are dilated using one drop of 10% Neo-Synephrine, 1% Mydriacyl, and 1% atropine. Only one eye of each animal in injected using an operating microscope. Fifty thousand, 100 000 or 250 000 cells in a total volume of 0.1 ml medium are injected into the posterior portion of the midvitreous through a 27-gauge needle 5 mm from the corneoscleral limbus at the superonasal quadrant. The test drug or the vehicle is injected as a second injection through the same entrance site and is delivered to the same place in the vitreous. Both injections have to be done slowly to prevent posterior retinal holes or deformation of the injected cells by shearing forces. The needle is withdrawn slowly to prevent leakage of vitreous from the wound. A paracentesis is performed to equilibrate the intraocular pressure after the second injection. An ointment containing antibiotics and atropine is applied at the end of the procedure.

EVALUATION

The development of proliferative vitreoretinopathy in the injected eyes is followed by indirect ophthalmoscopy; the appearance of membranes and detachment is documented by fundus photography.

MODIFICATIONS OF THE METHOD

A survey of eye models of inflammation including conjunctivitis, corneal responses to injurious stimuli, uveitis models, and retinal diseases has been presented by Woodward et al. (1989).

Retinopathy in **rats** resembling lesions seen in human retinopathy of prematurity could be provoked in newborn rats by exposure to 80% O_2 for the first 5 days of life (Ricci et al. 1995, 2000).

Hikchi et al. (1996) described an experimental model of ocular inflammation induced by intravitreal injection of interleukin 1 beta in rabbits.

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Chapter P
Pharmacological models in dermatology

P.1 Skin sensitization testing

P.1.1 Guinea pig maximization assay

PURPOSE AND RATIONALE

A variety of methods are available for the prospective identification of skin sensitizing chemicals. The methods that have been used are the Draize rabbit irritancy test (Draize et al. 1944; Phillips et al. 1972), the occluded guinea pig patch test (Buehler 1965), and the guinea pig maximization test (Magnusson and Kligman 1969; Magnusson 1980). The guinea pig maximization test is an adjuvant sensitization test requiring intradermal injections of the test substance, in combination with Freund's complete adjuvant, which stimulates non-specifically the immune system of treated animals, enhancing their ability to respond to sensitizing chemicals.

PROCEDURE

Groups of 15–20 guinea pigs (Hartley strain) are used. On day 0 an area of 4×6 cm over the shoulder region is clipped short with an electric clipper. Three pairs of intradermal injections are made simultaneously, so that on each side of the midline there are two rows of injections each. The injection sites are just within the boundaries of the 2×4 cm patch, which is applied one week later. Injections are (1) 0.1 ml Freund's adjuvant alone, (2) 0.1 ml test material, and (3) 0.1 ml test material in Freund's adjuvant. Control animals are given the same injections but without the test agent, i.e. Freund's adjuvant and vehicle.

On day 7, the same area over the shoulder region is again clipped and shaved with an electric razor. The test agent in petrolatum is spread over a 2×4 cm filter paper in an even, rather thick layer or, if liquid, to saturation. The patch is covered by an overlapping, impermeable plastic adhesive tape. This in turn is firmly secured by an elastic adhesive bandage, which is wound around the torso of the animal. The dressing is left in place for 48 h. Control animals are exposed to the vehicle without the test agent in the same way as the experimental group.

On day 21, in experimental and control animals the flanks are shaved on both sides on an area of 5×5 cm each. Filter paper pieces, 2×2 cm, are sealed to the flanks for 24 h with the same occlusive bandage as for topical induction: (1) left side: patch with the test agent in the highest non-irritant concentration. The same vehicle as for topical induction is used; (2) right side: path with the vehicle.

On day 23, reading is made 24 h after removing of the patches. By then, skin irritation due to the occlusive dressing has usually faded.

EVALUATION

If the challenge reactions in the experimental group clearly outweigh those in the control group, the agent is regarded to be a sensitizer. To grade the substances according to the percentage of animals sensitized, the substances are divided into 5 classes, ranging from weak (grade I) to extreme (grade V) sensitizers (Kligman 1966).

MODIFICATIONS OF THE METHOD

Improvement of the classical guinea pig maximization test was proposed by several authors: Maurer et al. (1980), Shillaker et al. (1989), Botham (1992), Kashima et al. (1993), Basketter et al. (1995), Frankild et al. (1996), Vohr et al. (2000), Steiling et al. (2001).

A predictive assay for contact allergens using human skin explant cultures has been described by Pistoor et al. (1996).

Use of **transgenic animals** to investigate drug hypersensitivity has been proposed by Moser et al. (2001).

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P.1.2
Popliteal lymph node hyperplasia assay

PURPOSE AND RATIONALE

The popliteal lymph node assay in mice or rats has been recommended as a tool for predicting allergies (Kammuller et al. 1989; Bloksma et al. 1995; Koch et al. 2000; Pieters 2001). Moreover, the inhibition of popliteal lymph node hyperplasia can be measured (Schorlemmer et al. 1998; Mollison et al. 1999). The test can be used to study compounds potentially effective in allergic eczema.

PROCEDURE

Spleens from Brown-Norway rats are harvested aseptically, splenocytes expressed by compression with a hemostat in Dulbecco's phosphate buffered saline (DPBS), red blood cells lysed with Tris (0.16 M) buffered in ammonium chloride (0.17 M) buffer, washed twice (400 *g*), irradiated (20 Gy), washed in DPBS, and suspended in DPBS at 5×10^7 cells per ml. On day 0, recipient Lewis rats are injected subcutaneously into the plantar surface of the right hindpaw with 0.1 ml of the splenocyte suspension. Compounds are dissolved in an appropriate vehicle and dosed daily, 2 ml/kg, on days 0–3. Recipients are sacrificed on day 4 and popliteal lymph nodes (PLN) from both hind limbs from vehicle control rats, or the right popliteal lymph node from drug-treated rats, are dissected free and weighed individually on a microbalance (Mollison et al. 1993). The average weight of PLN from the left leg of vehicle-treated animals is used as background.

EVALUATION

Percent inhibition is calculated using the following formula:

$$100 - \frac{PLN_{\text{exp.}} - PLN_{\text{mean left}}}{PLN_{\text{mean right}} - PLN_{\text{mean left}}} \times 100$$

- $PLN_{\text{exp.}}$ = experimental PLN weight
- $PLN_{\text{mean left}}$ = mean vehicle control left PLN weight
- $PLN_{\text{mean right}}$ = mean vehicle control right PLN weight

ED_{50} values are derived by simultaneous least square regressive analysis.

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P.1.3 Local lymph node assay

PURPOSE AND RATIONALE

More recently, the local lymph node assay in mice has been recommended for measurement of allergenic potency (Kimber and Weisenberger 1989; Kimber et al. 1995; Kimber 2001; Basketter and Scholes 1992; Basketter et al. 2001).

PROCEDURE

Groups of mice (CBA strain), weighing 20–25 g, receive topical applications of the test chemical on the dorsum of both ears, once a day for three consecutive days. In standard analyses, three concentrations of the test material are evaluated together with the relevant vehicle control. Five days after the initiation of exposure, all mice receive an intravenous injection of [^{3}H]-labeled thymidine into their tail vein. Five hours later, animals are sacrificed and draining auricular lymph nodes excised. A single cell suspension of lymph node cells is prepared by gentle mechanical disaggregation and the cells are washed and resuspended in trichloroacetic acid for at least 12 h at 4 °C. Precipitates are resuspended in trichloroacetic acid and transferred to an appropriate scintillation fluid. The incorporation by draining lymph node cells of [^{3}H]-labeled thymidine is measured by ß-scintillation counting and recorded as mean disintegrations per min (dpm).

EVALUATION

For each concentration of the test material a stimulation index (*SI*) is derived relative to the concurrent vehicle control. Those chemicals that at one or more test concentrations induce a *SI* of three or greater are classified as skin sensitizers.

Dose-response curves are plotted in order to provide information on the relative potencies of skin sensitizers. The concentration of the test chemical required to produce a stimulation index (*SI*) of three (named *EC3* value) is calculated using the formula:

$$EC3 = c + [(3 - d) / (b - d) \times (a - c)]$$

where the data points lying immediately above and below the *SI* value of three on the dose-response plot have the coordinates (a,b) and (c,d), respectively.

MODIFICATIONS OF THE METHOD

On the basis of a modified local lymph node assay, Horney et al. (1997) analyzed immunosuppressive effects of topically applied drugs. On four consecutive days, NMRI mice were treated on the dorsal surfaces of both ears with increasing concentrations of test compound. During the last three days, the mice received in addition the contact sensitizer, oxazolone (1%). On day 5, draining auricular lymph nodes were removed in order to assess lymph node cell counts and perform flow cytometric analysis of lymph cell subpopulations.

Hariya et al. (1998) developed a non-radioactive endpoint in a modified local lymph node assay.

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P.2 Experimental dermatitis

Several methods involving skin reactions are discussed in other chapters, such as:

- Ultraviolet erythema in guinea pigs (H.3.2.2.1),
- Vascular permeability (H.3.2.2.2),
- Oxazolone-induced ear edema (H.3.2.2.4),
- Croton-oil ear edema in rats and mice (H.3.2.2.5),
- Spontaneous autoimmune diseases in animals (I.2.2.1),
- Passive cutaneous anaphylaxis (I.2.2.4),
- Arthus type immediate hypersensitivity (I.2.2.5),
- Delayed type hypersensitivity (I.2.2.6),
- Reversed passive Arthus reaction (I.2.2.7),
- Acute graft versus host disease (I.2.2.15),
- SLE-like disorder in MRL/lpr mice (I.2.2.16),
- Inhibition of allogenic transplant rejection (I.2.2.20).

P.2.1 Spontaneous dermatitis

P.2.1.1 NC/Nga mouse as model for atopic dermatitis

The NC/Nga mouse has been recommended as a model for atopic dermatitis (Matsuda et al. 1997; Tsudzuki et al. 1997; Suto et al. 1999; Vestergaard et al. 1999, 2000; Kotani et al. 2000; Aioi et al. 2001; Kohara et al. 2001). When kept in specific pathogen free conditions, it remains healthy, but when kept in non-sterile conditions, it spontaneously develops a disease resembling atopic dermatitis at the age of 6–7 weeks. The level of IgE in the blood gradually increases to very high levels, and peaks at the age of 16–18 weeks (Matsuda et al. 1997). This enhanced IgE production has been attributed to an increased sensitivity of the B cell to the CD40 ligand and to IL-4, which is the result of enhanced phosphorylation of Janus kinase 3, a feature found also in atopic dermatitis (Matsumoto et al. 1999). At 16–18 weeks, the mice develop dry skin, and, gradually, nodular lesions, which in turn become crusted wounds. The lesions are pruritic and they are located on the back, the neck, the ears and the face. Biophysical parameters show impairment of water retention and barrier function. The amount of ceramide in the skin decreases significantly (Aioi et al. 2001).

Histologically, the skin lesions in the NC/Nga mice are characterized by hyperkeratosis and parakeratosis, which resemble the lichenified lesions observed in atopic dermatitis patients. In the dermis, an infiltration is found, similar to that seen in atopic dermatitis patients, containing lymphocytes, eosinophils, mast cells and macrophages, in addition to a large population of dendritic cells (Vestergaard et al. 1999).

The lesions in the NC/Nga mouse improve after treatment with tacrolimus hydrate (FK506) ointment (Hiroi et al. 1998) and also with topical steroids (Vestergaard et al. 1999). Both treatments reverse the changes in the skin, block the expression of inflammatory cytokines and decrease the serum levels of IgE.

FURTHER DERMATITIS MODELS

Further animal models for atopic dermatitis were described, such as the NOA (Naruto Research Institute Otsuka Atrichia) mouse (Natori et al. 1999; Watanabe et al. 1999).

Barton et al. (2000) reported that mice lacking the transcription factor ReIB develop T cell-dependent skin lesions similar to human atopic dermatitis.

Herz et al. (1998) developed a human-SCID mouse model to analyze the possible role of bacterial superantigens in human allergic immune responses under *in vivo* conditions.

Atopic dermatitis-like symptoms were reported in hypomagnesaemic hairless rats by Neckermann et al. (2000).

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P.2.1.2 Motheaten mice

Mice homozygous for the autosomal recessive motheaten (me) or the allelic viable motheaten (me^v) mutations develop severe and early-age onset of systemic autoimmune and inflammatory disease (Green and Shultz 1975; Shultz et al. 1984; Shultz 1988; Kovarik et al. 1994; Su et al. 1998). These mice show arthritis, patchy dermatitis and hemorrhagic pneumonitis; the latter is considered to be the cause of the early death of me and me^v mice at the age of 3 and 9 weeks, respectively.

Homozygous me^v mice are identified first at the age of 3–4 days by focal depigmentation of the skin, followed by patchy absence of hair and by necrotic lesions on paws, tail and ears.

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P.2.1.3 Spontaneous erythema in hairless rats

The hairless rat (WBN/Kob-*Ht*) is a dominant mutant derived from the Wistar strain. With an incidence rate of about 4% in both male and female animals, at an age of 20 weeks an erythema appears spontaneously on the dorsal skin, gradually becoming widespread and progressive in nature (Tani et al. 1998). Histopathologically, erythema is characterized by dermatitis induced by an immunological reaction. Areas of erythema in the skin were decreased by treatment with dexamethasone (1 mg/kg) or cyclosporine (25 or 50 mg/kg) injected subcutaneously every other day for 2 weeks. The results suggested that erythema on the hairless rat could be used as an animal model of spontaneous dermatitis.

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P.2.2 Contact dermatitis

P.2.2.1 Skin reaction in mice

PURPOSE AND RATIONALE

The phenomenon of contact hypersensitivity in animals is thought to mirror atopic dermatitis or eczema in patients (Corsini et al. 1979; Cooper 1994; Leung 1997).

PROCEDURE

Mouse contact hypersensitivity

CD1 mice are sensitized on the shaved abdomen with 20 µl of 0.5% 2,4-dinitrofluorobenzene (DNFB) in a vehicle consisting of 95% acetone/5% olive oil on days 0 and 1. On day 5, groups of 10 mice are challenged with 0.2% DNFB with or without co-dissolved drug, 10 µl on both the internal and external surface of both ears. Mice are sacrificed 24 h post-challenge, and a 7 mm diameter circle punched from each ear is weighed immediately on a microbalance.

EVALUATION

The mean ear plug weight from naïve mice challenged with 0.2% DNFB are used as background control.

Percent inhibition is calculated using the following formula:

$$100 - \frac{\text{plug}_{\text{exp.}} - \text{plug}_{\text{mean naïve}}}{\text{plug}_{\text{mean sens.}} - \text{plug}_{\text{mean naïve}}} \times 100$$

- $\text{plug}_{\text{exp.}}$ = experimental ear plug weight
- $\text{plug}_{\text{mean naïve}}$ = mean naïve ear plug weight
- $\text{plug}_{\text{mean sens.}}$ = mean sensitized vehicle control plug weight

ED_{50} values are derived by simultaneous least square regressive analysis.

MODIFICATIONS OF THE METHOD

Lowe et al. (1977) used oxazolone-sensitized Swiss Webster **mice** to evaluate anti-inflammatory properties of a prostaglandin antagonist, a corticosteroid and indomethacin in experimental contact dermatitis.

Further studies in mice on allergic contact dermatitis were performed by Ek and Theodorsson (1990), Fraginals et al. (1990), Katayama et al. (1990), Trenam et al. (1991), Stanley et al. (1991), Maguire (1996).

Grabbe et al. (1995) showed that removal of the majority of epidermal Langerhans cells by topical or systemic steroid application enhances the effector phase of contact hypersensitivity in BALB/c mice.

Koyama et al. (1998) used tenascin-C knockout mice and studied the effect on dinitrofluorobenzene-induced dermatitis.

A spontaneous mutation characterized by chronic proliferative dermatitis in C57BL mice was described by HogenEsch et al. (1993).

Guinea pig contact hypersensitivity

Guinea pigs, six per group, are sensitized on the dorsal surface of one ear pinna with 50 µl of 10% DBFB in 50% acetone: 50% olive oil on day 0, and then on the opposing ear on day 1. On day 5, animals are shaved and challenged with 0.5% 1-chloro-2,4-dinitrochlorobenzene (DNCB) with or without co-dissolved drug, 15 µl per site, 4.8 µl per cm^2, on the dorsolateral surface (Hsieh et al. 1996). Naïve animals are challenged with DNCB and serve as nonspecific controls. The response is scored visually in a blinded fashion 24 h after challenge (0: no change; 0.5: questionable erythema; 1: faint or scattered erythema; 2: mild, confluent erythema; 3: moderate erythema without edema or induration; 4: strong erythema with uniform induration or edema; 5: severe erythema with induration or edema, plus ulceration). Data are calculated as for mouse contact hypersensitivity.

Boyera et al. (1992) tested repeated application of dinitrochlorobenzene to the ears of sensitized guinea pigs as an animal model for contact eczema in humans.

The effect of a topical preparation of mycophenolic acid on experimental allergic contact dermatitis of guinea pigs induced by dinitrofluorobenzene was described by Shoji et al. (1994).

Contact hypersensitivity in pigs

Groups of 6–12 pigs are sensitized with 10% DNFB in acetone/DMSO/olive oil (45 : 5 : 50 by volume) to the shaved outer aspect of both ears and bilateral sites of the lower abdomen, 100 µl per site, on day 0, with a second application of 5% DNFB to the internal pinna and the lower thorax on day 3. On day 9, pigs are restrained on a webbed canvas cart and the test area carefully shaved with an electric clipper. A pilot challenge with 0.1, 0.15, and 0.2% DNCB in acetone/olive oil (95 : 5 by volume), 3.8 µl per cm^2, is used to determine conditions for obtaining submaximal average response in each animal cohort. Pigs are scored by a blinded observer, 24 h post-challenge on a scale from 0 to 4 (0: no change; 0.5: questionable erythema; 1: faint or scattered erythema; 2: moderate erythema without induration or edema; 3: strong erythema with focal areas of edema or induration: 4: extreme erythema with uniform induration or edema). Pigs having a mean DNCB control score <1.5 are excluded (Hsieh et al. 1997). Based on their pilot response on day 10, animals are stratified into two groups having comparable mean scores, challenged on duplicate sites with DNCB in 95 : 5, with or without co-dissolved drug, and scored on day 11. Scores for each challenge site are compared with the average of the control spots treated with DNCB alone on the same pig, and expressed as percentage inhibition.

Bilski and Thomson (1984) recommended allergic contact dermatitis in the domestic pig as a model for evaluating the topical anti-inflammatory activity of drugs and their formulations.

Vana and Meingassner (2000) described morphologic and immunohistochemical features of experimentally induced contact dermatitis in **Göttingen minipigs**.

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P.2.2.2 Non-immunologic contact urticaria

PURPOSE AND RATIONALE

Contact urticaria is defined as a wheal-and flare reaction, appearing shortly after certain substances contact intact skin and disappearing within some hours, leaving normal appearing skin. Contact urticaria is divided into two main types, immunologic and non-immunologic (Maibach and Johnson 1975). Immunologic contact urticaria is mediated at least partially by specific IgE antibodies attached to mast cell membranes. Vasoactive substances, released from mast cells, elicit erythema and edema of the skin. Non-immunologic contact urticaria appears only on the contact area without previous sensitization. Specific antibodies against the causative substance are not found in serum. Lahti and Maibach (1984) investigated the suitability of the guinea pig for studies on non-immunologic contact urticaria.

PROCEDURE

Female Hartley strain guinea pigs weighing 350–500 g are used. Fifty µl of test substances is applied with a micropipette to both sides of the earlobe. One ear of the animal is challenged with the contact urticant, while the other ear serves as control with ethyl alcohol. Earlobe thickness is measured three times on four different sites using a string micrometer with round touching plates, 6 mm in diameter. The string of the instrument is adjusted so that the moving plate does not squeeze the tissue, but stops at once when it reaches the surface of the ear. The mean of 12 measurements is recorded as the pre-application thickness. All measurements after application of the test substance are performed once on the same four sites, and the mean is recorded as the post-application thickness.

The thickness of the ear is measured 5 min after application, and then every 10 min during the first h, every 15 min during the second, and every 30 min during the third h.

EVALUATION

The differences between post-application and pre-application values are recorded and plotted as time-response and dose-response curves.

MODIFICATIONS OF THE METHOD

Lauerma et al. (1997) used the trimellitic anhydride-sensitive mouse as an animal model for contact urticaria. BALB/c mice were sensitized with trimellitic anhydride by topical applications and treated with glucocorticosteroids, antihistaminics, or non-steroidal anti-inflammatory drugs. Ears were challenged with trimellitic anhydride and ear thickness was measured at baseline and 1, 2, 4, 8, and 24 h after challenge. Trimellitic anhydride caused a biphasic ear swelling response. However, there was also an early swelling by trimellitic anhydride in non-sensitized mice, suggesting that non-immunological as well as immunological mechanisms contribute to early swelling by trimellitic anhydride.

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P.3 Pruritus models

P.3.1 Studies in cpdm/cpdm mice

PURPOSE AND RATIONALE

Pruritus is a common feature of many skin disorders. Since its pathogenesis in largely unknown, proper treat-

ment is not available. The development of new treatments is hampered by the lack of animal models for studying pruritus (Woodward et al. 1985). Kuraishi et al. (1995) have proposed that pruritogenic but not algesiogenic agents stimulate the scratching activity in mice. Scratching is usually registered by counting the number of scratches from direct visual observation or from a video recording (Gmerek and Cowan 1983; Larsen et al. 1994; Thomas et al. 1994). Elliott et al. (2000) developed a method, which allows the automated registration of the scratching activity of the hind legs of mice for periods longer than 24 h.

PROCEDURE

Mice with chronic proliferative dermatitis (cpdm/cpdm mice), a spontaneous mutation of C57BL/Ka mice showing a skin disorder accompanied by severe scratching (Gijbels et al. 2000), at an age of 6–12 weeks or normal C57BL/Ka mice treated subcutaneously with 100 µg/mouse compound 48/80 or 3 mg/mouse histamine hydrochloride, are used.

Metal rings made of soft 1-mm-diameter aluminum wire are placed around both hind legs of the animal just above the ankle. The ring has to be of sufficient diameter that it is just free enough to rotate around the limb. Mice are housed individually in cages ($12 \times 18 \times 13$ cm), which are placed on a scratch detection unit consisting of a plastic outer casing containing the circuit board, and, inset into the top, four ferrite rods with copper coils.

As the mouse scratches, the movement of the metal rings (fitted around its hind legs) in the field generated by the coils elicits a signal that can be transformed into peaks of different frequencies using fast Fourier transformation. Data are downloaded to memory every 3 s, filtered and a power spectrum constructed. The power spectrum is analyzed for a scratch pattern every 1.5 s. Scratching is routinely classified as a signal that gives rise to peaks with maximal amplitudes of around 200 mV and frequencies greater than 15 Hz. The amplitude of general motor activity peaks (movement from bedding using hind limbs, drinking from water bottle) is usually greater than 500 mV and at frequencies of less than 10 Hz. In order to obtain the best discrimination between scratching and general motor activity, any contribution to the scratch frequencies of high energy/low frequency peaks is filtered out by setting the upper detection limit at 0.5 mV for scratching.

EVALUATION

Data are expressed as mean ±SEM Statistical analysis is performed by one-way analysis of variance followed by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Larsen et al. (1994) studied the influence of ultraviolet irradiation on scratching behavior in hairless mice. Especially the wavelengths 315–330 nm were more itch-provoking than erythemogenic.

Sugimoto et al. (1998) studied the effects of histamine H_1 receptor antagonists on compound 48/80-induced scratching behavior in mice.

Inagaki et al. (1999, 2000) studied the participation of histamine H_1 and H_2 receptors in passive cutaneous anaphylaxis-induced scratching behavior in ICR mice and evaluated anti-scratch properties of drugs in BBLB/c, ICR, and ddY mice treated with dinitrofluorobenzene painting.

Ko and Naughton (2000) described an experimental itch model in **Rhesus monkeys**.

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P.4 Psoriasis models

P.4.1 General considerations

Several authors reviewed the experimental models for psoriasis (Krueger and Jorgensen 1990; Boehncke 1997; Nickoloff 1999; Rosenberg et al. 1999; Schön 1999). In addition to studies in animals, various *in vitro* investigations were carried out with autopsy material from human psoriatic lesions. Several studies were devoted to the T-cell hypothesis of psoriasis (Bos and de Rie 1999). Lymphocytes may change epidermal growth homeostasis, leading to increased keratinocyte proliferation and abnormal differentiation of these apoptotic cells that end their life cycle as corneocytes.

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P.4.2 *In vitro* studies with isolated cells

P.4.2.1 Cultured keratinocytes

PURPOSE AND RATIONALE

Cultured keratinocytes have been used to study the pathogenesis of psoriasis (Mils et al. 1994; Bata-Csörgö et al. 1993, 1995a,b; Ockenfels et al. 1996; Nylander-Lundqvist and Egelrud 1997a,b; Nylander-Lundqvist et al. 1998; Konger et al. 1998; Szabo et al. 1998; Dimon-Gadal et al. 2000; Karvonen et al. 2000; Segaert et al. 2000; Ting et al. 2000) and to evaluate antipsoriatic drugs (Chapman et al. 1990; Ockenfels et al. 1995; Medalie et al. 1996; Lin et al. 1999; Diaz et al. 2000; Farkas et al. 2001). Sampson et al. (2001) tested the *in vitro* keratinocyte antiproliferant effect of *Centella asiatica* extract and triterpenoid saponins.

PROCEDURE

The medium (FDMEM) consists of Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin and 2% streptomycin.

SVK-14 keratinocytes (Taylor-Papadimitriou et al. 1982) are cultured in FDMEM medium in flasks at 10% CO_2 and 37 °C. When confluent, cells are washed with magnesium- and calcium-free phosphate buffered saline (PBS). PBS is decanted and cells detached by adding 5 ml of trypsin (0.05%) in EDTA (0.02%). PBS is added to a volume of 50 ml. The cell pellet obtained by centrifugation (1 000 *g*, 5 min) is re-suspended in 10 ml of FDMEM. Cell counts in the final suspension are determined using a haemocytometer, and the cell density is adjusted to 25 000/ml with FDMEM.

For microtiter assays, cells (5 000 in 200 µl medium) are inoculated into the inner wells of 96-well plates. The outer edge wells of the plate contain 200 µl of 10% fetal bovine serum in PBS. After 24 h at 37 °C, 10% CO_2, plating medium is replaced with FDMEM containing test material or standard in 200 µl. Standard substances are: madecassoside (50 nM–5 mM), asiaticoside (100 nM–10 mM), dissolved in methanol, and dithranol (0.33–170 µM) dissolved on DMSO. Each sample concentration is tested with 6 replicates on each of three separate plates. Cells exposed to FDMEM alone provide 100% growth control. Cells are incubated for 7 days at 37 °C, 10% CO_2 prior to carrying out the sulphorhodamine B assay.

For the sulphorhodamine B (SRB) assay (Skehan et al. 1990), cells are fixed by layering 100 µl of ice-cold 50% trichloroacetic acid on top of the growth medium. Cells are incubated at 4 °C for 1 h, after which plates are washed 5 times with cold water, excess water drained off and the plates left to dry in air. SRB stain (50 µl; 0.4% in 1% acetic acid) (Sigma) is added to each well and left in contact with the cells for 10 to 30 min after which they are washed with 1% acetic acid, rinsing 4 times until only the dye adhering to the cells is left. The plates are dried and 100 µl of 10 mM Tris buffer added to each well to solubilise the dye. The plates are shaken gently for 5 in on a plate reader and the absorbance is read at 550 nm using a Titertek Multiscan MCC/340 II plate reader.

EVALUATION

Mean optical density (OD ±SD) is calculated for each concentration from the six replicate wells in a single plate. The data is used to plot a dose response curve from which IC_{50} values ±SD are obtained.

MODIFICATIONS OF THE METHOD

Fogh et al. (1993) described an *ex vivo* skin model. Keratomized psoriatic skin samples were incubated in the presence of the calcium ionophore a23 187 and

arachidonic acid for 45 min at 37 °C. After extraction of lipids, eicosanoids were determined by reversed-phase high-performance liquid chromatography in combination with specific radioimmunoassays.

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P.4.2.2 Effect on T lymphocytes

PURPOSE AND RATIONALE

Psoriasis vulgaris is considered as an inflammatory cell-mediated autoimmune disorder (Uyemura et al. 1993; Gottlieb et al. 1994; Bata-Csörgö et al. 1995; Austin et al. 1999). Lesional skin T cells contribute pro-inflammatory/type 1 (T1) cytokines to initiate and maintain the cell-mediated keratinocyte hyperplasia in inflammatory lesions. Atopic dermatitis is characterized by the presence of Th2 cell and their respective cytokines (Cooper 1994; Leung 1997). A therapeutic agent down-regulating both Th1 and Th2 cytokine production would be desirable (Mollison et al. 1999; Santamaria et al. 1999).

PROCEDURE

Human mixed leukocyte reaction (MLR) assay

Fifty milliliters of sodium heparinized blood, obtained from normal, unrelated donors, is mixed with an equal volume of Dulbecco's phosphate buffered saline (DPBS). Peripheral blood mononuclear cells (PBMC) are isolated by density centrifugation at 400 *g* over Histopaque-1 077. A sufficient number of PBMC are washed three times in RPMI-1 640 medium and used as responder cells. The remaining PBMC (stimulator cells) are washed as above and treated with 25 μg/ml mitomycin C for 30 min at 37 °C in an atmosphere of 5% CO_2 and 100% humidity and then washed three times with RPMI-1 640 medium. The stimulator cells are pooled at $0.5–1.0 \times 10^6$ cells per ml per donor in RPMI-1 640 medium. Cells are cultured in medium consisting of RPMI-1 640 supplemented with 4 mM L-glutamine, 500 μM 2-mercaptoethanol, 10% fetal bovine serum, 25 units/ml penicillin, and 25 μg/ml streptomycin. Mixed leukocyte reactions are performed in 96 well flat-bottom plates (Corning, Acton MA) in a final volume of 220 μl, containing 20 μl of culture medium with or without test compound or standard (ascomycin, purified from a fermentation of *Streptomyces hygroscopicus* ssp. *ascomyceticus* ATCC 14 891 and characterized according to Or et al. 1993), 100 μl of responder cells (1×10^6 cells per ml) and 100 μl of stimulator cells ($2–4 \times 10^6$ cells per ml). Cultures are incubated at 37 °C in an atmosphere of 5% CO_2 and 100% humidity for 4 days. On day 4, 0.5 μCi of [^{3}H]thymidine is added to each well during the last 6 h of culture. Cultures are harvested onto glass-fiber mats using a 96 well harvester. [^{3}H]Thymidine uptake is measured by direct β-counting using a Matrix 9 600 β-counter (Packard, Meriden, CT).

Inhibition of IL-2 production in human whole blood and PBMC

Fifty milliliters of venous blood from normal donors is drawn into tubes containing sodium heparin. Twenty-five milliliters of blood is used directly. PBMC are isolated from the remaining sample as described above and cultured at 1×10^6 cells per ml with supplemented RPMI-1 640. Whole blood or PBMC are induced to secrete IL-2 with 50 ng PMA per ml plus 1 μg ionomycin per ml. Immunosuppressive potency of test compound vs. standard is determined by measuring the inhibition of IL-2 secretion. Assays are performed in 96 well flat-bottom plates in a volume of 210 μl, which includes 190 μl of whole blood or PBMC (1×10^6 cells per ml), 10 μl of PMA (1 μg/ml) and ionomycin (20 μg/ml) mixture and 10 μl of serially diluted test compound. Plasma or tissue culture supernatants are collected 24 h later and IL-2 concentrations determined by ELISA.

Porcine MLR assay

Fifty milliliters of sodium heparinized venous blood is drawn from pigs and diluted with an equal volume of 0.9% saline. PBMC are isolated by density centrifugation for preparation of responder and stimulator cells as described above. MLR are performed as described for human MLR, using 100 μl of responder cells (2×10^6 cells per ml) and 100 μl of stimulator cells ($0.5–4 \times 10^6$ cells per ml).

Rat MLR assay

Popliteal, inguinal, and mesenteric lymph nodes from newly sacrificed Lewis rats and spleens from Brown Norway rats are aseptically removed. Single cell suspensions of splenocytes and lymphocytes are prepared using forceps and a hemostat to macerate the tissues. Red blood cells in the splenocyte suspension are lysed by 2 min incubation at ambient temperature in red blood cell lysis buffer containing 0.14 M NH_4Cl in 0.0167 M Tris-HCl, pH 7.2. Responder cells from Lewis rat lymph nodes and stimulator cells from Brown Norway rat spleens are washed three times in RPMI-1 640 medium and then sedimented at 400 *g* for 10 min. Stimulator cells are prepared as for human, and the assay is conducted in serum-free AIM-V medium supplemented with 1% Antibiotic-Antimycotic solution and 50 μM 2-mercaptoethanol, using an optimized cell ratio of 100 μl of responder cells (2×10^6 cells per ml) and 100 μl of stimulator cells ($1–2 \times 10^6$ cells per ml) and an incubation period of 4 days.

Mouse MLR assay

Spleens are aseptically removed from newly sacrificed C_3H(C_3H/HeNCrIBR) and BALB/c mice. Splenocytes are prepared and the assay conducted as for rat, using an optimized cell ratio of 100 μl of responder cells (4×10^6 cells per ml) and 100 μl of stimulator cells (1×10^6 cells per ml).

Concanavallin A-induced guinea pig lymphocyte proliferation assay

Spleens are aseptically removed from newly sacrificed guinea pigs. Single cell suspensions of splenocytes are prepared as for rat using 4 min incubation in lysis buffer to remove red blood cells. Splenocytes are washed three times in RPMI-1 640 medium and adjusted to 6.25×10^5 cells per ml in culture medium consisting of RPMI-1 640 supplemented as described for human MLR. Concanavallin A-induced proliferation reactions are performed in a volume of 200 μl, containing 20 μl of culture medium with or without test compound or standard, 160 μl of guinea pig splenocytes and 20 μl concanavallin A (20 μg/ml). Cultures are incubated for 3 days, pulse labeled on the last day with [^{3}H]thymidine, harvested and counted as described.

Inhibition of cytokine secretion

Serially diluted test compound or standard is added to 96 well plates with PBMC in RPMI-1 640 medium at 1×10^6 cells per ml. PMA and ionomycin are added to effect 10 ng per ml and 500 ng per ml concentrations, respectively. Supernatants from these cultures are collected 24 h later after centrifugation and stored at –80 °C until use. Concentrations of IL-2 and IFN-γ in the supernatants are determined by ELISA. For assessing inhibition of IL-4 and IL-5 secretion in T cells by the test compound, $CD4^+$ cells are isolated from PBMC using a T cell subset enrichment column (RandD Systems, Minneapolis, MN). Purified $CD4^+$ cells are cultured with serially diluted test compound and PMA at 10 ng per ml in plates previously coated with 100 ng per ml solution of anti CD3 antibody (Immunotech, Westbrook, ME). Supernatants are collected after centrifugation 40 h later. IL-4 and IL-5 concentrations in the supernatants are determined by ELISA.

EVALUATION

Dose response curves for test compound and standard are established and IC_{50} values for inhibition calculated.

MODIFICATIONS OF THE METHOD

Gillitzer et al. (1996) studied neutrophil migration in psoriatic lesions as a model for neutrophil chemotaxis.

Kunstfeld et al. (1997) investigated the migration of inflammatory T cells from psoriasis through superficial vascular plexus and through deep vascular plexus endothelium. Superficial and deep plexus human skin was placed separately into SCID mice.

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P.4.3
Psoriasis models in normal animals

P.4.3.1
Mouse tail model for psoriasis

PURPOSE AND RATIONALE

The mouse-tail test was introduced by Jarrett and Spearman (1964). The model is based on the induction of orthokeratosis in those parts of the adult mouse-tail, which have normally a parakeratotic differentiation. Quantification of orthokeratosis was achieved by morphometric evaluation of the scales with the aid of a semiautomatic evaluation unit (Bosman et al. 1992; Bosman 1994; Sebök et al. 1996).

PROCEDURE

Male albino NMRI mice weighing 25–27 g are used. The tails are treated locally with 0.1 ml ointment applied to the proximal part of the tail. For the contact time of 2 h a plastic cylinder is slipped over the tail and fixed with adhesive tape. At the end of contact time the cylinders are removed and the tails washed. Animals are treated once daily, 5 times a week, for 2 weeks. Five to 8 animals are used per dosage group. Two hours after the last treatment the animals are sacrificed and the tails prepared histologically (fixation in 4% formalin, paraplastic embedding). Longitudinal sections of about 5 µm thickness are prepared and stained with hematoxylin-eosin.

The epidermal thickness is measured as the distance between the dermo-epidermal borderline and the beginning of the horny layer. Five measurements per animal are made in each of 10 scales. Out of these 50 measurements the mean for the individual animals is calculated.

The sections are examined for the presence of a granular layer or isolated granular cells induced in the previously parakeratotic skin areas (10 sequential scales per animal). The measurements are carried out at the border of the scale with a semiautomatic image evaluation unit (VIDS III, AI TEKTRON). The distances are obtained in pixels (1 pixel = 1.2120 μm).

Quantitative values of orthokeratosis are obtained by measuring the length of the granular layer per scale (A) and the whole scale length (B). The whole scale length is defined as the length of the scale lying between two adjacent hair follicles, beginning and ending at the turning point between hair follicle and scale. Percent keratosis is calculated by the formula:

$$(A / B) \times 100$$

EVALUATION

Ten sequential scales per animal are measured and the results given in % orthokeratosis per scale. Five to 8 animals are taken for one drug concentration or control group. Thus 50–80 individual orthokeratosis values are obtained per test group. Mean and standard error of the mean are calculated per animal and per group. From the individual orthokeratosis values per dosage group (50–80 scales) a frequency distribution is constructed. Therefore the values (ranging from 0 to 100% orthokeratosis) are grouped into classes with a constant class interval of 10% (class 1: 0–10%; class 2: 10.1–20%; class 10: 90.1–100% orthokeratosis). The frequency per class is calculated in %:

$$\text{class frequency} = \frac{\text{no. of scales in the class}}{\text{total no. of scales}} \times 100$$

For every class the cumulative frequency is constructed by adding the frequencies of all foregoing classes.

Due to a non-Gaussian distribution of the orthokeratotic values (100% is the maximal effect), the Mann-Whitney U-test is used.

The efficacy of test compounds on epidermal differentiation is calculated from the mean length of orthokeratosis after treatment with the substrate (Ok_s) and with salicylic acid as control (Ok_c) using the formula:

$$(Ok_s - Ok_c) / (100 - Ok_c) \times 100$$

MODIFICATIONS OF THE METHOD

Nagano et al. (1990) studied the effect of tumor necrosis factor in the mouse-tail model of psoriasis.

Beyaert et al. (1992) induced a psoriasiform inflammatory reaction in mice by subcutaneous injection of a combination of tumor necrosis factor and lithium chloride.

Several studies were performed in **guinea pigs** (Miller and Ziboh 1990; Kumar et al. 1992; Maini et al. 1999). Tuzun et al. (1993) reported psoriasis lesions in guinea pigs receiving propranolol; however, their results could not be confirmed by Wolf et al. (1994).

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P.4.3.2 Rat ultraviolet ray B photodermatitis model for psoriasis

PURPOSE AND RATIONALE

Rat ultraviolet ray B photodermatitis has been proposed as an experimental model of psoriasis vulgaris by Nagakuma et al. (1995).

PROCEDURE

Male Wistar rats weighing around 300 g are used. Hair on the dorsal skin is clipped and carefully shaved. An area (1.5 × 2.5 cm) on one side of the flank is irradiated for 15 min (1.5 J/cm^2) at a vertical distance of 20 cm with UV-B lamps. A biphasic erythema is observed. Immediately after irradiation, initial faint erythema appears, disappearing within 30 min. The second phase of erythema starts 6 h after the irradiation and gradually increases, peaking between 24 and 48 h. The color is brownish-red, and the reaction is confined to the exposed area with a sharp boundary. By 48–72 h after irradiation, dark-brown scale is formed on the erythematous lesion. Pieces of the scale are relatively thick. The scale separates and the erythema decreases daily. The skin sites return to normal about 10 days after irradiation.

The irradiated rats are sacrificed after various time intervals by decapitation under ether anesthesia. Skin biopsies are taken immediately, fixed in 10% formalin and embedded in paraffin. Tissue sections (4 μm thick) are stained with hematoxylin and eosin. The numbers of the keratinocyte layers, including the basal layer, are counted by direct microscopy.

For DNA labeling (Morimoto et al. 1991), 20 mg/kg 5-bromo-2-deoxyuridine (BrdU) is administered i.p. 8 h before decapitation. Frozen tissue sections (8 μm) are prepared with a cryostat. The sections are then fixed in acetone for 10 min, 4% paraformaldehyde containing 1% $CaCl_2$ (pH 7.0) for 10 min and 1% glutaraldehyde for 5 min. After fixation, sections are digested with 0.006% pepsin in 10 mM HCl for 10 min at 37 °C, and incubated in 4 M HCl for 30 min at ambient temperature to denature DNA. They are then incubated with anti-BrdU mouse monoclonal antibody and then with alkaline phosphatase-conjugated anti-mouse IgG sheep IgG antibody. Antibody-binding sites are visualized with naphthol and fast red at pH 9 in the dark. The number of labeled as well as unlabeled cells at the epidermal basal layer or outer root sheath cell layers of the hair follicles are counted in five high-power fields to a total of at least 100 cells. The intensity of BrdU incorporation is demonstrated as a labeling index (%) with the following formula:

$$\text{labeling index} = \frac{\text{no. of labelled cells}}{\text{total cell no. counted}} \times 100$$

EVALUATION

Chronological changes of thickness of keratinocytes at the epidermis and the hair follicles as well as of DNA labeling indices are plotted as time-response curves.

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P.4.4 Psoriatiform skin diseases in spontaneous mice mutations

PURPOSE AND RATIONALE

Nearly 100 mouse mutations have been described as causing some type of abnormality of the skin or hair. These include "asebia", a mildly hyperkeratotic disorder with sebaceous gland hyperplasia, "ichthyosis", an example of abnormal hair growth associated with hyperkeratosis, "rhino" and "hairless", two related examples of congenital follicular malformations, and "flaky skin", a potential animal model of eruptive psoriasis.

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P.4.4.1 Flaky skin (*fsn*) mouse

PURPOSE AND RATIONALE

Flaky skin (*fsn*) is an autosomal recessive mouse mutation that causes pathologic changes in the skin, yielding a papulosquamous disease resembling human psoriasis (Sundberg et al. 1993). In addition, this mutation, assigned to distal chromosome 17, causes anemia and gastric forestomach hyperplasia (Beamer et al. 1995).

The *fsn* mutation arose spontaneously in the A/J inbred strain at The Jackson Laboratory (Beamer et al. 1986). Psoriasiform skin lesions are first evident as focal epidermal hyperplasia and inflammation at 2 weeks of age. These lesions become confluent and diffuse by 3–4 weeks of age and are associated with marked dermal infiltration of lymphocytes and small numbers of neutrophils and macrophages. Mast cell numbers increase significantly in the dermis from 2 weeks of age onward. Diffuse dermal neovascularization accompanies these cutaneous changes (Sundberg et al. 1997). Systemic lesions in-

clude progressive and massive papillomatosis of the stratified squamous epithelium of the forestomach, hyperplasia and dysplasia of the glandular stomach, increased apoptosis of cecal enterocytes, renal glomerulopathy associated with inflammatory cell infiltrates and fibrosis around the portal triads in the liver, splenomegaly due to massive erythropoiesis, and granulomatous lymphadenitis.

Scanning microscope examination (Morita et al. 1995) reveals a greatly thickened epidermis, and sparsity of hairs and scale accumulations in the epidermal surface. Hair shafts have conspicuous pits, striations, and exophytic protrusions. Nails are bent at a 90-degree angle with surface irregularities and accumulation of scale at the nail base. Transmission electron microscopic examination shows increased epidermal thickness, mitochondrial aberrations, and intraepidermal invasion by neutrophils. Keratin abnormalities are detected using immunocytochemical staining for profilaggrin. At the dermal-epidermal junction, numerous macrophages and mast cells are seen in close proximity to focal dissolutions of the basement membrane. A high density of collagen fibers and cellular infiltrates are evident in the papillary dermis.

Besides lymphadenopathy and mast cell accumulation, elevated serum IgE levels (>7 000 fold increase compared with normal littermates), autoimmunity (evidenced by glomerulonephritis with immune complex deposition in the kidneys), are observed in flaky skin mutant mice (Pelsue et al. 1998).

Peripheral lymph nodes of adult mutant (*fsn/fsn*) mice were found to contain almost 10-fold more leukocytes than peripheral lymph nodes from phenotypically normal littermates. Analysis of peripheral lymph node cells using mAbs and flow cytometry revealed that this predominantly lymphoid hyperplasia is characterized by approximately equivalent increases of $CD3^+$ T cells and $C19^+$ B cells (Abernethy et al. 2000a). A dysregulated expression of CD69 and IL-2 receptor alpha and beta chains was found on $CD8^+$ T lymphocytes (Abernethy et al. 2000b). Expression and function of IL-1 beta is increased in psoriasiform skin lesions of flaky skin (*fsn/fsn*) mice (Schön et al. 2001).

However, neutrophils also played a critical role of for the generation of psoriasiform skin lesions in flaky skin mice (Schön et al. 2000). Intraperitoneal injection of the neutrophil-depleting RB6-85C monoclonal antibody resulted in a dramatic reduction of the epidermal thickness.

Similar to active human psoriatic lesions, an increase of epidermal growth factor receptors was observed in *fsn/fsn* mice (Nanney et al. 1996).

Sundberg et al. (1994a) transplanted full-thickness skin grafts from flaky skin mice on the dorsal skin of genetically athymic nude (*nu/nu*) mice. The grafts maintained the psoriatiform phenotype of the donors.

Backcrosses to different mouse strains suggested several modifier genes affecting the *fsn* phenotype (Sundberg et al. 1994b). As cyclosporine A, in contrast to glucocorticoids, was not effective when used for topical or systemic treatment of *fsn* lesions, it seems that there is no immunologic basis for these lesions. Therefore, it remains uncertain whether the flaky skin mouse can be used to test potential therapeutic compounds.

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P.4.4.2 Asebia (*ab/ab*) mouse

PURPOSE AND RATIONALE

The asebia mouse was first described by Gates and Kasarek (1965) as hereditary absence of sebaceous glands. The asebia mouse represents a spontaneous mutation in BALB/c mice leading to hyperplasia of the epidermis and chronic inflammatory dermal changes, including enhanced cellularity, edema and elevated mast cell numbers (Josefowicz and Hardy 1978; Brown and Hardy 1988). The circadian rhythms in cell proliferation are suppressed in the chronically hyperproliferative epidermis of the asebia mouse (Brown et al. 1988a). UVB radiation as well as anthralin and tar with UVB further stimulate proliferation in the already hyperproliferative epidermis of the asebia mouse (Brown et al. 1988b, 1989). The gene for the enzyme stearyl-CoA desaturase 1, which is expressed in sebaceous glands, is disrupted in the asebia mouse (Parimoo et al. 1999; Zheng et al. 1999; Miyazaki et al. 2000). Besides Sdc1 and Sdc2, Zheng et al. (2001) identified Scd3 – a novel gene of the stearoyl-CoA desaturase family with restricted expression in the skin. Since T cell and neutrophil infiltrates are not observed in asebia mice, a pathogenesis distinct from psoriasis has been suggested (Schön 1999).

Asebia-2J (Scd1(ab2J)): a new allele and a model for scarring alopecia was described by Sundberg et al. (2000).

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P.4.5 Psoriasiform skin diseases in genetically modified animals

PURPOSE AND RATIONALE

Based on expression in inflammatory skin disorders, several factors, such as cytokines, are considered to play a crucial role in the pathogenesis of psoriasis and other skin diseases. Transgenic animals are tools to analyze these factors (Meyer 1990; Rothnagel et al. 1990; Sellheyer 1996; Rosenberg et al. 1999).

Bullard et al. (1996) described a polygenic mouse model of psoriasiform skin disease in **CD18-deficient mice**. CD18 deficiency in patients results in recurrent microbial infections, leukocytosis, impaired wound healing, failure of granulocyte emigration, and lack of pus formation (Anderson et al. 1995). A hypomorphic mutation for CD18 was introduced by Wilson et al. (1993) into mice with homocygotes displaying mild leukocytosis, an impaired response to chemically induced peritonitis, and delays in transplantation rejection. When this CD18 mutation was crossed back onto PL/J strain of mice, the development of an inflammatory skin disorder was observed (Bullard et al. 1996). The disease is characterized by erythema, hair loss, and the development of crusts. The histopathology reveals hyperplasia of the epidermis, subcorneal microabscesses, orthohyperkeratosis, parakeratosis, and lymphocyte exocytosis similar to human psoriasis and other hyperproliferative skin disorders. The dermatitis rapidly resolved after subcutaneous administration of dexamethasone.

Dermal infiltrates of macrophages/monocytes within the dermis of clinically uninvolved skin were seen in transgenic mice with epidermal overexpression of K14/IL-1α, suggesting a role of IL-1α for macro-

phage attraction (Groves et al. 1995). In severely affected animals, inflammation reactions occur that are characterized by a mixed inflammatory infiltrate, and by acanthosis and parakeratosis. **IL-1α transgenic mice** support a primary role of IL-1α as an inducer of cutaneous inflammation, which may be helpful for clarifying pathogenesis of psoriasis.

Wilson et al. (1990) reported that expression of the **BNLF-1 oncogene** of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6.

Cook et al. (1997, 1999) found that overexpression of the heparin-binding EGF-related ligand amphiregulin in the epidermis of transgenic mice induces a psoriasis-like cutaneous phenotype. **Transgenic mice with a K14 enhancer/promoter-driven amphiregulin gene** targeted to the epidermis displayed a macroscopic phenotype that included extensive areas of scaling and erythematous skin with marked alopecia. Histological examination revealed hyperkeratosis, focal parakeratosis, acanthosis, mixed leukocytic infiltration that included both CD3-positive T cells in the dermis and epidermis, and a tortuous vasculature.

Two completely opposite phenotypes were observed in **mice expressing K10/BMP-6** (bone morphogenetic protein-6, a member of the TGF-β superfamily) within the epidermis (Blessing et al. 1996). Whereas keratinocyte proliferation was severely reduced in animals with strong and homogenous expression of the transgene, weaker and patchy expression led to marked hyperproliferation.

Rodriguez-Villanueva et al. (1998) reported that **human keratin-1.bcl-2 mice** aberrantly express keratin 6, exhibit reduced sensitivity to keratinocyte cell death induction, and are sensible to skin tumor formation.

The concept of vascular endothelial growth factor (VEGF) being an important angiogenic factor in psoriatic skin (Detmar et al. 1994) was supported by **transgenic mice with constitutive epidermal K14/VEGF expression**. These animals exhibited dilated and contorted dermal microvessels (Detmar et al. 1998). There was also an increased number of dermal mast cells, and leukocyte adhesion and extravasation was enhanced in VEGF transgenic mice.

Klement et al. (1996) found that **IκBα deficiency** results in a sustained NF-κB response and widespread dermatitis in mice.

Seitz et al. (1998) reported that alterations on NF-κB function in transgenic epithelial tissue demonstrate a growth inhibitory role for **NF-κB**.

To determine the role of κB-α deficient immunocytes in the pathogenesis of skin disease in adult mice, Chen et al. (2000) utilized the RAG2-deficient blastocyst complementation system to generate **RAG2–/–, IκB-α–/– chimeras**. These animals display a psoriasiform dermatitis characterized by hyperplastic epidermal keratinocytes and dermal infiltration of immunocytes, including lymphocytes. Skin grafts transferred from diseased chimeras to recipient nude mice produce hyperproliferative epidermal keratinocytes in response to stimulation.

Transgenic mice overexpressing suprabasal integrins α_2, α_5, and β_1 demonstrate a phenotype similar to psoriasis with cycles of flaking and inflamed skin, suggesting that disrupted keratinocyte integrin-ligand interactions may play a role in hyperproliferative states (Carroll et al. 1995).

Robles et al. (1996) found that **expression of cyclin D1 in epithelial tissue** of transgenic mice results in epidermal hyperproliferation and severe thymic hyperplasia.

Carroll et al. (1997) used the **involucrin promoter to overexpress IFN_γ** in the suprabasal layers of transgenic mouse epidermis. The mice exhibited striking hypo-pigmentation of the hair due to reduction of DOPA-positive melanocytes. Severely affected mice had reddened skin, growth retardation, hair loss, and flaky skin lesions. The skin was characterized by a dermal infiltrate of T lymphocytes and macrophages/monocytes.

When **scid/scid mice were reconstituted with MHC-matched, but minor histocompatibility mismatched $CD4^+/CD45RB^{hi}$ T lymphocytes**, almost all of the animals developed skin lesions similar to human psoriasis within 4–8 weeks after transfer (Schön et al. 1997). The psoriatiform skin lesions did not develop in recipients of unfractionated splenocytes or $CD4^+/CD45RB^{lo}$ T cells, indicating that T cell dysregulation is the primary pathogenic factor in this model. When recipients of $CD4^+/CD45RB^{hi}$ T cells were treated with either cyclosporine A or UVB irradiation, the psoriasiform lesions were dramatically improved demonstrating that immunosuppressive therapies are efficacious. The model was proposed as a T cell initiated murine model of inflammatory skin lesions (Schön and Parker 1997).

Mann et al. (1993) found that mice with a **null mutation of the TGF-a gene** have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation.

Turksen et al. (1992) described psoriasiform changes in the skin of transgenic mice with **overexpression of interleukin 6**.

Guo et al. (1993) reported that targeting expression of **keratinocyte growth factor** to keratinocytes elicits striking changes in epithelial differentiation in transgenic mice.

Hong et al. (1999) showed that IL-12, independently of IFN-gamma, plays a crucial role in the pathogenesis of a murine psoriasis-like disorder. Only a few *scid/scid* mice develop skin lesions when $CD4^+$/$CD45RB^{hi}$ are transferred alone. Coadministration of LPS plus IL-12 or staphylococcal enterotoxin B in *scid/scid* mice one day after **$CD4^+$/$CD45RB^{hi}$ cell transfer** greatly enhances disease penetrance and severity.

Schön et al. (2000) reported a cutaneous inflammatory disorder in **integrin alpha E (CD103)-deficient mice**. Skin inflammation correlated with alpha E deficiency in mice with a mixed 129/Sv×BALB/c background.

Transgenic rats expressing human HLA-B27 and β_2-microglobulin develop psoriasiform skin changes as part of a multi-organ inflammatory disease (Hammer et al. 1990). In the most severely affected lines, psoriasiform lesions first occurred at about 20 weeks of life and were observed in 10% to 80% of animals. Male appeared to be more prone to skin changes than females (Taurog et al. 1993, 1994, 1999). Similar to human pathogenesis, T cells seem to be the most important factor in HLA-B27 transgenic rats (Breban et al. 1996).

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P.4.6 Xenotransplantation of human psoriatic skin

PURPOSE AND RATIONALE

Xenotransplantation models for psoriasis involve the transfer of human psoriatic skin to animals. First studies were performed by transplantation of involved psoriatic and non-psoriatic human skin onto congenitally athymic (nude) mice (Krueger et al. 1975, 1985; Baker et al. 1992). More recently, human psoriatic skin grafts were transplanted onto SCID mice (Nickoloff et al. 1995; Boehncke et al. 1994, 1997, 1999; Gilhar et al. 1997; Sugai et al. 1998; Dam et al. 1999; Raychaudhuri et al. 2001) allowing screening of anti-psoriatic drugs.

PROCEDURE

Keratome biopsies ($7 \times 2 \times 0.05$ cm containing both dermis and epidermis) are obtained from clinically symptomless skin of patients with psoriasis or from psoriatic plaques. Prior to the procedure, the skin is defined and infiltrated with 1% lidocaine and epinephrine 1 : 200. The full-thickness skin biopsy is dissected into 12 grafts. CB-17 SCID mice at an age of 6–8 weeks are anesthetized with an i.p. injection of 1.56 mg phenobarbital before transplantation of the human xenografts on the flank area. The grafts are sutured with absorbable 6-0 Vicryl Rapid suture and covered with Xeroform dressings for one week. Animals transplanted with the psoriatic plaque are then randomized into groups receiving PBS as negative controls or 0.15 mg cyclosporine A or the test compound intradermally into the xenografts. Within 4 weeks, the animals are sacrificed by CO_2 asphyxiation and 4 mm punch biopsies are obtained from each xenograft. Biopsies are fixed in 10% neutral-buffered formalin for paraffin embedding.

EVALUATION

A semiquantitative scale is used to indicate the extent of new vessel formation (angiogenesis) that can be seen between the xenograft and the underlying fascia muscularis. A calibrated eyepiece microscope is used for estimating the epidermal thickness in the vertical sections. Statistical differences are calculated using Student's *t*-test for multiple comparisons.

MODIFICATIONS OF THE METHOD

Nickoloff et al. (1995) studied severe combined immunodeficiency mouse and human psoriatic skin chimeras. Autologous blood-derived $CD4^+$ cells injected into symptomless transplanted psoriatic skin engrafted onto SCID mice produced full-fledged psoriatic lesions (Wrone-Smith and Nickoloff 1996; Nickoloff and Wrone-Smith 1999; Nickoloff et al. 1999; Nickoloff 2000).

Yamamoto et al. (1998) studied the effects of superantigen-driven peripheral blood mononuclear cells on the persistence of psoriasiform epidermis and on the cytokine gene expression of grafted psoriatic skin. Staphylococcal enterotoxin B-stimulated peripheral blood mononuclear cells from psoriatic patients were repeatedly injected under the grafted full-thickness involved psoriatic skin onto severe combined immunodeficient mice. A persistence of the psoriasiform epidermis was found after 5 weeks. E-selectin expression was observed on endothelial cells in the upper epidermis of the mice.

Tissue specificity of E- and P-selectin ligands in Th1-mediated chronic inflammation was studied by Chu et al. (1999).

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P.5
Scleroderma models

P.5.1
Scleroderma models in chicken

Scleroderma is an autoimmune disorder which occurs in the severe systemic form, a localized scleroderma (morphoea) and as lichen sclerosus et atrophicus. Chickens of the University of California line 200 (**UCD-200 chickens**) developed an inherited inflammatory fibrotic disease, closely resembling human progressive systemic sclerosis (scleroderma) (Gershwin et al. 1981; Van de Water et al. 1984; Boyd et al. 1991; Gruschwitz et al. 1991, 1993; Herold et al. 1992; Needleman 1992; Brezinscheck et al. 1993; Ausserlechner et al. 1997; Nguyen et al. 2000). An acute inflammatory stage started at an age of about 60 days after hatching, leading to fibrosis with fast progression with severe lymphocytic infiltration and excessive accumulation of collagen in skin and internal organs. A sequential increase of type VI, type I and type II procollagen transcripts and a progressive increase of autoantibodies to histone, to ssDNA and to dsDNA was found.

A further strain of chickens with progressive systemic sclerosis (UCD 206) has been identified (Duncan et al. 1995; Sgonc et al. 1995).

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P.5.2 Scleroderma models in mice

The **tight-skin mouse** is another experimental model for scleroderma (Walker et al. 1990; Muryoi et al. 1992; Delany and Brinkerhoff 1993; Kasturi et al. 1993, 1994; Wallace et al. 1994; Pablos et al. 1995; Frondoza et al. 1996). This mutant mouse develops autoantigens specific for scleroderma target tissues. The tight-skin disease was first discovered in 1967 at the Jackson Laboratory in the B10.D2(58N)/Sn mouse strain. The disease occurred spontaneously. Green et al. (1976) have identified the tsk locus and shown it to be linked to pallid locus. The tight skin character is transmitted as an autosomal dominant trait and homozygous tsk/tsk mice die *in utero*. The striking feature of the disease is the presence of thickened skin, firmly bound to subcutaneous and deep muscular tissue, with excessive accumulation of collagen in skin and internal organs (Menton et al. 1978; Osborn et al. 1983). Synthesis and accumulation of type I collagen is markedly increased (Jimenez et al. 1986; Ong et al. 1998). Biochemical studies have shown that the prolyl hydroxylase and glycosaminoglycan content of the affected tissue is increased (Ross et al. 1987).

Additionally, the **tight skin 2 mouse** has been described as an animal model of scleroderma displaying cutaneous fibrosis and mononuclear cell infiltration (Christner et al. 1995, 1996; Wooley et al. 1998; Sgonc et al. 1999).

Bleomycin-induced scleroderma in genetically mast cell deficient WBB6F1-W/W^V mice was described as an animal model of sclerotic skin by Yamamoto et al. (1999a–c).

Murine sclerodermatous graft-versus-host disease is considered as a model for human scleroderma (Claman 1990; Schiltz et al. 1994; McCormick et al. 1999).

A syndrome resembling human systemic sclerosis (scleroderma) in **MRL/lpr mice lacking interferon-receptor** was described by Le Hir et al. (1999).

A **mouse model for vitiligo** was described by Lerner et al. (1986), designated C57BL/6J-vit/vit. The vitiligo mouse has congenital dorsal and ventral white spots as well as progressive replacement of pigmented hairs with each spontaneous molt or after plucking.

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P.6
Pemphigus models

P.6.1
Experimentally induced pemphigus in mice

PURPOSE AND RATIONALE

Pemphigus is defined as a group of spontaneously occurring disorders of skin and mucosae in which blisters and erosions form within the epidermis due to loss of intercellular contact (Marks 1987). Pemphigus occurs also spontaneously in domestic animals, such as dogs, cats, and horses (Hurvitz 1980; Scott et al. 1983; Sueki et al. 1997). Various attempts were made to study experimentally induced pemphigus in mice (Buschard et al. 1981; Takahashi et al. 1985; Anhalt et al. 1986a,b; Rock et al. 1990; Juhasz et al. 1993; Koch et al. 1997; Fan et al. 1999; Mahoney et al. 1999; Amagai et al. 2000; Zillikens et al. 2001).

Fan et al. (1999) screened four strains of female mice (BALB/c, DBA/1, SJL/J, and HRS/J) for their ability to produce pathogenic anti-desmoglein 3 antibodies. Only BLB/c mice immunized with full length desmoglein 3 can produce pathogenic antibodies capable of causing acantholysis in human foreskin in culture and blistering in neonatal mice.

PROCEDURE

Six- to 8-week-old female BALB/c, DBA/1, SJL/J, and HRS/J are immunized four times with 20 µg/mouse of purified desmoglein 3 protein in CFA (on days 1, 10, 20, and 30), four times with 20 µg/mouse of extracellular domain of desmoglein 3 (on days 40, 50, 60, and 70), and twice with 20 µg/mouse of refolded desmoglein 3 (on days 80 and 90), then they are boosted twice more with 20 µg/mouse of extracellular domain of desmoglein 3 in IFA by i.p. inoculation (on days 97 and 104). Control groups of mice are similarly immunized with BSA.

IGs from 10 ml of pooled serum from mice immunized with desmoglein 3 are precipitated with 40% ammonium sulfate, dialyzed against PBS twice, lyophilized, and reconstituted in water to 1 ml. One hundred µl of the reconstituted antibodies/mouse is injected into neonatal BALB/c mice.

EVALUATION

Mice are examined 18–24 h post injection for blister formation. Cross sections containing the blister and comparable areas in control animals are biopsied and frozen sections are prepared for routine histological examination.

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P.7
Ichthyosis vulgaris models

P.7.1
Experimentally induced ichthyosis in mice

PURPOSE AND RATIONALE

Ichthyosis vulgaris is a heterogeneous autosomal skin disease characterized by dry, scaly skin, mild hyperkeratosis, and a decreased or absent granular layer that either lacks, or contains morphologically abnormal, keratohyalin granula (Anton-Lamprecht and Hofbauer 1972). Both the skin of ichthyosis vulgaris patients and keratinocytes cultured from affected individuals exhibit reduced or absent profilaggrin mRNA and protein levels (Sybert et al. 1985). The symptoms and the genetics of the ichthyotic (*ic/ic*) mouse were described by Spearman (1960), Green et al. (1974), Jensen and Esterly (1977), Holbrook (1989). Presland et al. (2000) demonstrated loss of normal profilaggrin and filaggrin in flaky tail (*ft/ft*) mice and proposed this as an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris.

Elias et al. (1983) and Chung et al. (1984) induced ichthyosis in the hairless mouse by treatment with diazacholesterol and used this as an assay for comparative potency of topical retinoids.

PROCEDURE

Male hairless mice, 2–3 months old, are fed either a normal laboratory diet supplemented with 60 mg/kg/day of 20,25-diazacholesterol or normal laboratory diet. Diazacholesterol blocks the conversion of Δ^{24}-reduction of desmosterol to cholesterol (Anderson and Martt 1965), and as a result desmosterol accumulates in stratum corneum lipids rather than cholesterol. Ichthyotic changes generally become apparent after 8–12 weeks and are most pronounced over the back and tail. With the exception of some reduction in body weight in comparison with controls, the animals appear healthy. Since the tail manifests the most exaggerated scaling, this site is used for topical drug applications. As the animals become ichthyotic, the daily dose of diazacholesterol can be lowered to 30 mg/kg and maintained at that level.

The test substances (retinoids) are first solubilized in a small volume of dimethyl sulfoxide and then dissolved in Cremophore EL. A volume of ~100 µl of each test substance is applied once daily to circumscribed areas of the tail. Treatment groups consist of 3 animals each, and each animal serves as its own control. The drop is first placed on an investigator's gloved index fingers and then spread evenly around a designated band of the tail. Each animal is treated with two concentrations of the test drug. The most proximal and most distal portions of the tail are left untreated as control regions. Applications are continued for 2 weeks. At three- or four-day intervals, and at the termination of the experiment, the clinical response is graded from 0 to 4+, with 0 indicating no response and 4+ indicating removal of all visible scale, leaving a glistening surface.

Prior to biopsy, the skin surface is coated with a thin film of flexible collodion to prevent fragmentation during frozen sectioning. Perpendicular sections of biopsy samples are stained with aqueous 8-anilino-2-naphthalene sulfonic acid, which on fluorescence microscopy depict selectively stratum corneum hydrophobic membrane domains.

EVALUATION

Both control and drug-treated sections are measured in a double-blind manner. The mean and SE from a minimum of five separate regions are tabulated. Significant differences are determined by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Harlequin ichthyosis (ichq): a juvenile lethal mouse mutation with ichthyosiform dermatitis was described by Sundberg et al. (1997).

Knox and Lister-Rosenoer (1998) described an infantile ichthyosis in **rats** and proposed this as a new model of hyperkeratotic skin disease.

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P.8
Xeroderma models

P.8.1
Experimentally induced xeroderma in mice

PURPOSE AND RATIONALE

Xeroderma pigmentosum is an autosomal recessive disorder characterized by hyperphotosensitivity and multiple cancers in association with abnormal DNA repair (Robbins et al. 1974; Satokata et al. 1992). Xeroderma pigmentosum group A (XPA) gene-deficient mice cannot repair UV-induced DNA damage and easily develop skin cancers by UV-irradiation (Nakane et al. 1995; Miyauchi-Hashimoto et al. 1996, 1999). Kuwamoto et al. (2000) tested the involvement of enhanced prostaglandin E_2 production in the photosensitivity in Xeroderma pigmentosum group A model mice and the influence of a prostaglandin synthesis inhibitor.

PROCEDURE

XPA gene-deficient homogeneous mice, age 8–12 weeks, are used (Nakane at al 1995). The UVB source consists of a bank of fluorescent sunlamps with an emission spectrum from 270 to 375 nm, peaking at 305 nm. The mice are anesthetized by i.p. injection of phenobarbital to keep them immobile during exposure. The ears are irradiated with 250 mJ per cm^2 of UVB. Immediately after irradiation, the test drug (20 µl of 1% solution of the prostaglandin synthesis inhibitor indomethacin) is applied to the ears. Ear thickness is measured with a dial thickness gauge immediately before irradiation and 1–4 days after irradiation. The amounts of PGD_2, PGE_2, and PGF_{2a} in mouse ears at 0, 24, 48, and 72 after UVB irradiation are determined by enzyme immunoassay.

EVALUATION

Student's *t*-test is employed to determine the statistical difference between means.

MODIFICATIONS OF THE METHOD

De Boer et al. (1999) described a mouse model for the DNA repair/basal transcription disorder trichothiodystrophy, revealing cancer predisposition.

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P.9 Acne models

P.9.1 Activity on sebaceous glands of rats

PURPOSE AND RATIONALE

Bioassays for topical antiandrogens are based on inhibition of sebum secretion. Sebum production is increased by endogenous or exogenous androgens in many species including humans. In the mouse (Lapière and Chèvremont 1953; Neumann and Elger 1966), the Mongolian gerbil (Mitchell 1965), and the golden hamster (Hamilton and Montagna 1950), the male sex hormone stimulates sebum production and sebaceous gland growth. Morphometric evaluation by light microscopy in the rat has shown that castration causes a large reduction in the volume of the glands (Sauter and Loud 1975). The administration of testosterone over several days produces an enlargement of the sebaceous glands. Early transformations, which take place in the morphology of the organelles in sebaceous cells, can be observed by electron microscopy. In the cytoplasm of intermediate cells a large number of vesicular elements derived from the smooth endoplasmic reticulum is formed, participating in the synthesis of lipids which appear as droplets of varying size (Karasek 1968; Morohashi 1968). Following an increase of lipid droplets, the cells increase in size, become totally differentiated and are pushed towards the apex of the gland where they break up and release their content (sebum) into the infundibulum. This effect is used for morphometric evaluation of topical anti-androgens.

PROCEDURE

Groups of 5 adult male Sprague-Dawley rats weighing 180–220 g are shaved in the interscapular area. Twenty-four hours later, the test preparation or the standard (cyproterone acetate) is applied locally to the shaved area at increasing doses (0.05, 0.5, and 5 mg/cm^2) in 20 µl ethanol. The treatment is continued for three weeks. Controls receive ethanol only. The animals are sacrificed 24 h after the last administration. Pieces of skin from the interscapular region are excised and processed for evaluation by electron microscopy. The volume density of the smooth endoplasmic reticulum vesicles is measured.

EVALUATION

Dose-response curves are established for volume density of vesicles of the smooth endoplasmic reticulum after various doses of antiandrogen and testosterone standard in order to calculate activity ratios. Statistical comparison with untreated controls allows calculation of threshold doses.

MODIFICATIONS OF THE METHOD

Ebling and Petrow (1993) tested 19-Aldehydo-4-androstene-3,17-dione, an estrogen precursor that inhibits sebaceous secretion in ovariectomized testosterone-treated rats.

De Young et al. (1984, 1985) described intradermal injection of *Propionibacterium acnes* as a model of inflammation relevant to acne. Intradermal injection of killed *P. acnes* into the rat ear induces a chronic acne-like inflammation characterized by edema and cell infiltration of several months' duration, formation of comedones, hypersensitization, and transepithelial elimination.

Shamoto et al. (1999) investigated the dermal histology and the regional draining superficial lymph node of a new mutant strain of hairless rats (ISh). The homocygote ISh rat was characterized as having naked and wrinkled skin. The comedo-like casts in the skin resembled human acne.

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P.9.2
Activity on sebaceous glands of the fuzzy rat

PURPOSE AND RATIONALE

Fuzzy rats, a genetic mutant between hairless and haired albino rats, exhibit sexual dimorphism in the skin (Ferguson et al. 1979). A brown-colored, thick seborrhoeic coating on the entire back is characteristic of male rats while the female rats show clear skin with white fuzzy hairs. In male rats the hair follicles resemble human sebaceous follicles associated with hyperplastic glandular lobules, dilatation and microcomedo (Plewig and Kligman 1975). Castration caused a reduction in size of the sebaceous glands and ducts, and a testosterone implant in castrated rats resurrected glandular hyperplasia and ductal dilatation (Uno et al. 1990).

This rodent model for androgen-dependent hyperplasia of the sebaceous glands is useful for the study of many pharmacological aspects, comprising the rate of percutaneous absorption, stability, and affinity to target organs of the testing compounds and selection of adequate vehicles for topical application (Ye et al. 1997).

PROCEDURE

Peripubertal male fuzzy rats at an age of 25 days weighing about 40 g are kept in single cages at a temperature of 24 °C and a 12-h light-and-dark cycle. They are divided into several groups for local treatment with 5α-reductase inhibitors or androgen receptor antagonists. One group serving as control is castrated at an age of 25 days in anesthesia. Approximately 0.5 ml of a 1% solution of test compounds dissolved in 30% propylene glycol, 50% alcohol and 20% water are applied to the other groups in a 4×4 cm area on the lower back once per day 5 days per week. Vehicle solution alone is applied to the control and castrate groups. After 8 weeks of treatment, the animals are euthanized by anesthesia with ketamine HCl, 100 g/ml, xylazine HCl, 120 g/ml, acepromazine, 5 g/ml, 1 ml/kg intramuscularly.

Photographs of the backs are taken pre-treatment and every 2 weeks after the start of treatment.

Eight weeks after starting treatment, the animals in all groups are given an intraperitoneal injection of 200 mg/kg bromodeoxyuridine 2 h before euthanasia. A blood sample is then collected from the right ventricle of the heart under anesthesia with the ketamine-xylazine-acepromazine mixture. After euthanasia with an overdose of the same anesthesia solution, fresh skin tissues are taken by a punch (4 mm diameter) from the marked area of the back and immediately incubated with EDTA solution for the split-skin preparation. The rest of the skin in the marked area is cut and fixed with 10% neutral buffered formalin solution for morphometric analysis of the sebaceous glandular lobules. The ventral lobes of the prostate are dissected and the weight is measured.

For morphometric analysis of the sebaceous glands, the size of sebaceous glandular lobules is first determined. Using formalin-fixed skin, small skin samples are taken by punch (4 mm diameter) and serial frozen sections (40 μm thick), horizontal to the epidermal surface, are cut and collected in water-filled wells. Four to five free-floating sections containing sebaceous glandular lobules are selected and stained with 1% osmium and 2.5% potassium dichromate solution for 2–3 min. The sections are washed with distilled water and mounted on a glass slide with aqua mount. Darkly stained globular lobules are distinctively seen under the microscope or on microvideo images. The largest lobular area in each glandular image is measured by a computer-assisted microimage apparatus, using a program of image analysis.

Split preparations of the pilosebaceous organ are used to measure the size of the sebaceous lobule and duct, and the number of DNA synthesis sebocytes. Fresh skin samples, 4 mm punched, are incubated with 17 mM EDTA in phosphate buffer (0.1 M, pH 7.4) for 2 h at 27 °C. The pilosebaceous organs attached to the epidermis are manually split from the dermis. Follow-

ing fixation with 10% buffered formalin, free-floating split tissues are stained by the immunocytochemical method, using a monoclonal antibody against bromodeoxyuridine with the avidin-biotin complex method.

On viewing the split tissue under a stereomicroscope, the sebaceous glandular lobules with a duct attached to the follicular shaft are clearly visible. After mounting on a slide glass, these *in situ* images of the sebaceous glands are observed on a computer monitor with a microvideo apparatus, and the area of the lobes, the diameter of the duct and number of bromodeoxyuridine-stained nuclei in the sebocytes are measured.

EVALUATION

Group data are expressed as means ±standard error. The results are analyzed using the *t*-test.

MODIFICATIONS OF THE METHOD

Marit et al. (1995) presented anatomical and physiological parameters of the fuzzy rat, collected for each sex at five ages, including histological and clinical biochemical profiles, organ and body weights, and a characterization of gross and histopathological findings.

Yourick and Bronaugh (2000) studied percutaneous penetration and metabolism of 2-nitro-*p*-phenylendiamine in human and fuzzy rat skin.

Salcido et al. (1995) described an animal model and computer-controlled surface pressure delivery system for the production of pressure ulcers. A method for inducing dermal pressure lesions on the fuzzy rat was developed using a computer-controlled displacement column, which produced a constant tissue interface pressure.

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P.9.3
Activity on ear sebaceous glands of Syrian hamsters

PURPOSE AND RATIONALE

The ear sebaceous glands of the Syrian hamster have been proposed as a model system for human sebaceous glands because of the similarities in morphology and in turnover time (Hamilton and Montagna 1950; Plewig and Luderschmidt 1977). As an androgen-sensitive structure, the ear sebaceous glands have been used to determine the effect of antiandrogenic compounds by the use of histoplanimetry on projections of sagittal sections of the ear (Luderschmidt and Plewig 1977). Matias and Orentreich (1983) developed a stripped skin planimetric method to measure the ear sebaceous gland areas in Syrian golden hamsters.

Seki et al. (1995) determined the effects of topically applied spironolactone on androgen stimulated sebaceous glands in the hamster pinna.

PROCEDURE

Adult female Syrian golden hamsters, 9–10 weeks of age and 110–120 g in weight, are kept at constant temperature and humidity and fed commercially prepared hamster food and water. The hamsters are divided at random into three groups. Testosterone propionate (80 µg dissolved in 1 ml sesame oil) is administered to two groups every other day over a two weeks period to stimulate androgen responses. Hamsters in the third group are injected with 1 ml sesame oil only as controls. The hamsters receive once daily applications of 0.1 g of a colorless clear hydrophilic gel preparation of 5% spironolactone, containing ethanol and isopropyl alcohol on the ventral side of the right pinna for two weeks. The left side is left untreated as control. On day 15, 4 mm punch biopsy specimens from each hamster are obtained from the central region of the bilateral pinnae where sebaceous glands are most developed.

The sebaceous gland size is measured by the whole mount technique (Motoyoshi 1988). After the biopsy specimens are immersed in physiological saline at 4 °C for 18 h, cartilages are removed. Then, each specimen is immersed in 2 N NaBr at 37 °C for one hour, and the epidermis is peeled off with fine forceps. The obtained dermis sheet containing sebaceous glands is stained with Susan III and mounted on a glass slide.

EVALUATION

An area of five or more sebaceous glands, including all the sebaceous acini attached to one pilosebaceous unit in each biopsy specimen, is measured with a computerized image analyzer (Olympus CIA-102) and shown as a mean value ± standard deviation ($mm^2/100$). The values are compared using Student's paired *t*-test.

MODIFICATIONS OF THE METHOD

Gollnick (1990, 1992) evaluated azelaic acid proposed for acne treatment, using comedo formation in the hamster ear model and recommended it as a new substance in the spectrum of antiacne agents.

Matias and Gaillard (1995) studied the local inhibition of sebaceous gland growth in the ventral ear pinna of sexually mature male Syrian hamsters by topically applied androgen receptor inhibitors.

Seki and Morohashi (1993) investigated the effect of some alkaloids, flavonoids and triterpenoids on the lipogenesis of sebaceous glands of the hamster ear. Lipogenesis was assayed by determining ^{14}C incorporation into sebaceous lipids extracted from the sebaceous glands, which were incubated with ^{14}C-acetate.

Morgan et al. (1993) studied the occurrence of zinc-induced synthesis of metallothionein in skin after topical application of the anti-acne drug Zineryt® lotion in hamster ears. The dinitrophenyl hapten-sandwich immunohistochemical method involving a monoclonal anti-metallothionein antibody was used to detect and localize zinc-binding metallothionein in the treated and untreated hamster skin.

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P.9.4
Activity on ear sebaceous glands of rabbits

PURPOSE AND RATIONALE

Several authors used the rabbit ear model to study comedo formation (Weirich and Longauer 1974; Mills and Kligman 1975; Motoyoshi 1983a; Kligman 1989; Ito et al. 1985, 1991; Maeda 1991) in order to assess the comedogenicity of cosmetics, toiletries and drugs and to evaluate potential anti-acne drugs. Sebaceous follicles in the inner surface of rabbit ears are sensitive to many substances called comedogenes, which, when applied topically, induce comedo formation. This comedo induction takes place after about 2 weeks of repeated topical application of a chemical comedogen such as 1% coal tar, 50% oleic acid, or 50% tetradecane.

PROCEDURE

Male rabbits weighing 2.5–3.5 kg are used. Tetradecane, testosterone, and dimethyl sulfoxide are separately inuncted on rabbit pinnas once a day for 4 weeks. The pinnas are biopsied on days 1, 3, 7, and 28. Untreated pinnas and squalene-treated pinnas serve as controls. Three-dimensional images of sebaceous glands are reconstructed from their serial histological sections using a computer-image analysis system.

EVALUATION

The volumes of the sebaceous glands, the number of acini, and the volume of individual acini are compared between groups.

MODIFICATIONS OF THE METHOD

Motoyoshi (1983b) studied the correlation between surface microscopy and dermal histology in tetradecane-induced comedones in rabbit ear skin.

Kligman and Kligman (1994) used the rabbit ear to assay the comedogenic potentialities of an array of known tumorigens. Complete carcinogens and some tumor promoters were invariably strongly comedogenic at concentrations of 1.0% and below. The rabbit ear model was recommended to be an easy and reliable way to screen for carcinogenicity.

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P.9.5
Activity on the hamster flank organ

PURPOSE AND RATIONALE

The flank organs of Syrian golden hamsters are located on each flank of the animal consisting mainly of sebaceous tissue. Like sebaceous glands in other species, these pigmented spots respond to androgens by an increase in size. This proliferation is inhibited by systemical or topical anti-androgens.

PROCEDURE

Female Syrian golden hamsters (*Mesocricetus auratus*), weighing 80–110 g are kept at constant temperature on a commercial diet and water ad libitum. They are castrated 24 h prior to the experiment. The costovertebral region is shaved; the horny layer is stripped the day prior to the tes and then every 3 days. For stimulation, animals receive a subcutaneous dose of 250 µg testosterone propionate in 25 µl peanut oil in the dorsal neck fold, for three weeks (weekdays only or continually). On the same days, the anti-androgen dissolved in water/ethanol (1 : 4, v/v) is applied locally to the left flank organ at increasing doses, using a micropipette under a continuous air stream to enhance the evaporation of the solvent. At least 5 animals are used per group. Controls receive vehicle only, and another group, testosterone propionate only. After treatment the animals are sacrificed under ether anesthesia. The 2 major perpendicular axes of the pigmented spot overlying each flank organ are measured and multiplied to obtain the surface area index. The flank organs are excised and divided into two halves along the major axis, immediately fixed in 10% formalin and embedded in paraffin. The 5 µm-thick sections are stained with haematoxylin-eosin. Sebaceous glands and hairs are measured in the first 2 sections of each half of the specimen, using a semiautomatic computerized image analyzer. The sebaceous gland area in each field is quantified in square millimeters. The diameter of all the hair under each flank organ is measured in micrometers.

EVALUATION

The local (topical) anti-androgenic activity of the test compound is estimated by the ability to inhibit the effects in the ipsilateral treated flank organ whereas the systemic activity is evaluated by the inhibition on the untreated contralateral flank organ. The values for surface area index, sebaceous gland area, and average of diameter of the hairs on the flank organ of the treated left and the untreated right side are compared for anti-androgen-treated animals with controls using two-way analysis of variance and *t*-test.

MODIFICATIONS OF THE METHOD

Wuest and Lucky (1989) studied the differential effect of testosterone on pigmented spot, sebaceous glands and hair follicles in the Syrian hamster flank organ.

Noto et al. (1991) quantified the antiandrogenic activity of topically applied canrenoic acid in the hamster flank organ. The flank organs of female Syrian hamsters were stimulated by subcutaneous administration of testosterone propionate. Sebaceous glands and hair follicles were measured by a computerized image analyzer. Using this method, the same authors (Noto et al. 1992) compared the activity of some topical antiandrogens.

Aricò et al. (1993) found no antiandrogenic effects of topical bifonazole on sebaceous glands and hair in the hamster flank organ.

Lucky et al. (1995) studied the autoradiographic localization of tritiated dihydrotestosterone in the flank organ of the albino hamster.

Foreman et al. (1984) found that in the hairless hamster, progesterone can antagonize dihydrotestosterone-mediated hypertrophy of the sebaceous gland.

CRITICAL ASSESSMENT OF THE METHOD

The relevance of the hamster flank organ model to man has been challenged by Franz et al. (1989).

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P.9.6
Activity on the skin of the Rhino mouse

PURPOSE AND RATIONALE

The Rhino mouse has been widely used as an experimental acne model to evaluate topically active comedolytic and anti-keratinizing agents (Kligman and Kligman 1979; Ashton et al. 1984; Mezick et al. 1984; Chatelus et al. 1989; Bernerd et al. 1991; Bouclier et al. 1991; Tramposch et al. 1992; Zheng et al. 1993; Sundberg 1994; Fort-Lacoste et al. 1999).

Rhino mice are hairless mutants with a rhinoceros-like appearance, which carry the rhino gene, a recessive allele of the hairless gene ($hr^{rh}hr^{rh}$) (Howard 1941). This recessive mutation on chromosome 14 results in a mouse with wrinkled skin devoid of body hair by age of 25 days. At that time, the end of the first hair cycle, the follicular papillae fail to follow the regressing hair follicles and become isolated in the dermis. The papillae do not re-associate with the follicular epithelium to initiate a new hair follicle cycle. The upper remnants of the hair follicles are filled with sloughed, cornified cells and form utriculi with a small sebaceous gland at their base, resembling an open comedone. The Rhino skin becomes progressively loose, forming folds and ridges, due to the expansion of the surface, secondary to abortive hair follicles filling with cornified debris. The utriculi progressively enlarge, forming pillory cysts (pseudocomedones), which are dilated follicular infundibula filled with cornified debris (Mann 1971). The dermal cysts of the rhino mouse develop into unopened sebaceous glands (Bernerd et al. 1996).

Seiberg et al. (1997) studied the effects of trypsin on apoptosis, utriculi size and skin elasticity in the rhino mouse. González et al. (2000) measured the effect of graduated local doses of all-*trans* retinoic acid applied to the skin of rhino mice with fluorescence excitation spectroscopy and compared the data with histological findings.

PROCEDURE

Seven-weeks old female rhino mice ($hr^{rh}hr^{rh}$) obtained from Charles River Laboratories are divided into groups receiving graduated doses of test compound (all-*trans* retinoic acid) dissolved in an ethanol/propylene glycol mixture (70 : 30) or the solvent as controls. The solution is applied once per day in doses of 100 µl on the entire dorsal skin, for 5 consecutive days each week, for a total period of 2 weeks. Animals in all groups are sacrificed 24 h after the last treatment and biopsies are obtained immediately following sacrifice. Fluorescence excitation spectra are collected from dorsal skin on a daily basis during treatment. During the measurements, the animals are sedated with inhalation of methoxyflurane.

Fluorescence excitation spectra are obtained *in vivo* with a fluorescence spectrophotometer (SkinSkan, SPEX, Edison NJ) equipped with a 450 W Xenon lamp, double monochromators on the excitation and emission, a photomultiplier detector (R928P, Hamamatsu, Japan) connected to a single photon counting system and a bifurcated quartz fiber bundle (Model 1950; SPEX Ind., Edison, NJ) for light delivery and collection. The individual fibers are 100 µm in diameter and are randomly arranged to form a bundle 6 mm in diameter. The resolution is 4 nm, the intensity of the excitation radiation is in the range of 1–20 mW/cm^2, and the total delivered radiation dose is below the erythema threshold. Each fluorescence measurement consists of a set of eight serial excitation spectra collected by positioning the emission monochromator from 340 to 480 nm in increments of 20 nm and scanning the excitation monochromator from 260 to within 20 nm of the emis-

sion monochromator setting. Light from the excitation monochromator is focused into one leg of the bifurcated fiber bundle. The other leg of the fiber bundle is focused into the input of the emission monochromator. The joined end of the fiber bundle is brought into direct contact with the skin site measured. Care is taken to clean properly the fiber bundle end with an alcohol swab between measurements on different animals and that gentle pressure is applied to the animals during measurement.

Significant changes are observed in the fluorescence spectra as a result of the application of comedolytic agents. The first peak, located approximately at 295 nm, which is related to tryptophan, increases significantly, whereas the second peak at 340–370 nm, which is attributed to collagen links, exhibits a dramatic decrease.

For histology, skin samples approximately 5 mm in diameter are obtained from the skin of each animal following sacrifice, from the mid-line of the anterior portion of the skin. All specimens are fixed in 10% buffered formaldehyde for 24 h, and later embedded in paraffin. Tissue sections 5 µm thick are cut perpendicularly to the epidermal surface and stained with hematoxylin and eosin for light microscopic examination. For each animal, the epidermal and dermal thickness, utriculi diameter, and number of capillaries containing more than 5 erythrocytes are assessed with a micrometer eyepiece adapted to a microscope (Leitz SM-LUX, Ernst Leitz, Wetzlar, Germany) using a 40× objective for the measurement of epidermal and dermal thickness, and utriculi diameter, and a 20× objective for the count of dilated capillaries.

EVALUATION

Time-response curves and dose-response curves of the changes of fluorescence spectra are established compared with the changes of histological data.

MODIFICATIONS OF THE METHOD

In addition to image analysis, Seiberg et al. (1997) tested the effects of trypsin on skin elasticity and elastin expression in the Rhino mouse.

Beehler et al. (1995) studied gene expression of retinoic acid receptors and cellular retinoic acid-binding proteins in rhino and hairless mouse skin.

González et al. (1997) investigated DNA ploidy changes in rhino mouse skin after treatment with all-*trans* retinoic acid and retinol.

Petersen et al. (1984) developed an animal model using human face skin onto the nude mouse to study human sebaceous glands. The effects of androgens were evaluated.

Lesnik et al. (1992) reviewed agents that cause enlargement of sebaceous glands in hairless mice.

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P.9.7 Activity on the skin of the Mexican hairless dog

PURPOSE AND RATIONALE

The Mexican hairless dog has been recommended as a model for the comedolytic and morphogenic activity of retinoids and other anti-acne agents (Loux et al. 1974; Schwartzman et al. 1996; Kimura and Doi 1996). A semilethal mutation of an autosomal dominant 'L' gene produces an animal with multiple developmental defects, including poor dentition, early degeneration of the thymus, hairlessness and numerous comedones (Yankell et al. 1970a; González-Diddi et al. 1971; Goto et al. 1987; Fukuda et al. 1991). The Mexican hairless dog is nearly bald, with sparse, flimsy hair most abundant on the limbs and head. In most hairy species, the primary hair is surrounded by 7 to 10 accessory hairs. In this species, the follicles are mainly single and not in clusters. The skin is tan-colored. A brownish, waxy material unevenly coats the surface; this can be removed easily by soap and lipid solvents. A great number of comedones cover the surface, and these are nearly all of the open variety. The majority is rather small (a few millimeters in diameter), and may be viewed as horn-filled shallow invaginations. Scattered among these, especially on the face, neck and thighs, are larger, black-tipped open comedones. These are hard, deep, horny impactions, which are expelled with difficulty. A few small, closed comedones occur on the neck and lateral aspect of the chest.

Papulopustules are rare, and are found mainly on the metacarpus and metatarsus. They apparently do not develop from the rupture of comedones as in humans. Thus, this animal mimics human acne only with regard to comedones. These, unlike the human variety, do not originate from pre-existing sebaceous follicles. They arise de novo, and completely lack sebaceous glands.

PROCEDURE

About 1-year-old female animals are used. Areas of 4–5 cm^2 on the dorsolateral aspects of the trunk are treated once daily (5 working days) for 14 weeks with test formulations or tretinoin formulations (0.025–0.1%) as standard.

Biopsies are taken from each site under local anesthesia at 5 weeks and again at 14 weeks.

EVALUATION

The clinical picture and the histology of biopsies are compared before, during, and after treatment.

MODIFICATIONS OF THE METHOD

Yankell et al. (1970b) used the Mexican hairless dog for sunscreen recovery studies.

Hunziker et al. (1978) compared percutaneous penetration of benzoic acid, progesterone and testosterone between Mexican hairless dogs and man.

Matsumura et al. (1992) described a burn wound-healing model in the hairless descendant of the Mexican hairless dog.

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P.9.8 *In vitro* sebocyte model

PURPOSE AND RATIONALE

Cultured human sebocytes have been used by several authors to study sebum formation and to evaluate potential ant-acne drugs (Zouboulis et al. 1991a,b, 1993, 1994, 1998; Rosenfield 1989; Doran and Shapiro 1990; Doran et al. 1991; Akamatsu et al. 1993; Guy et al. 1996a,b; Rosenfield et al. 1998; Wauben-Penris et al. 1998; Tsukada et al. 2000; Fritsch et al. 2001).

PROCEDURE

Human sebaceous glands are isolated from facial skin and seeded on monolayer 3T3 cells (Xia et al. 1989). Primary sebocyte cultures are derived from the periphery of the gland lobules and are maintained to confluence before subcultivation. All experiments are performed using secondary sebocyte cultures, which consist of cells undergoing sebocytic differentiation.

Human sebocytes are seeded in 96-well culture plates at a concentration of 10^4 cells/well and are left to attach for 2 days at 37 °C with 5% CO_2 in culture medium consisting of Dulbecco's modified Eagle's medium and Ham's F12 medium (1 : 1) supplemented with 8% fetal calf serum, 2% human serum, 10 ng/ml epidermal growth factor, 10^{-9} M cholera toxin, 3.4 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The medium is then aspirated, and serum-free keratinocyte basal medium (KBM) (Clonetics, San Diego, CA) without additives, supplemented with steroids, e.g., testosterone (10^{-8}–10^{-5} M), 5a-dihydrotestosterone (10^{-8}–10^{-5} M), or spironolactone (10^{-12}–10^{-7} M) or their combinations are added to 6 wells at each concentration. KBM is concomitantly added to another 6 wells serving as controls. The plates are incubated at 37 °C for 10 days before evaluation. KBM with and without compounds is changed every 2 days.

Cell numbers of treated human sebocytes in 96-well culture plates are assessed over 9 days by counting single-cell suspensions in Neubauer chambers and compared with the absolute fluorescence units (AUF) of parallel wells obtained using the 4-methylumbelliferyl heptanoate (MUH)-fluorescence assay (Stadler et al. 1989). This assay is based on the hydrolysis of the fluorogenic substrate MUH by esterases of proliferating cells. A stock solution of 10 mg/ml MUH is prepared in DMSO and kept frozen at –20 °C until use. On the day of assessment, KBM is removed, and the cells are washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (pH 7.2). The MUH stock solution is diluted in phosphate-buffered saline up to 100 µg/ml, and 100 µl of the final solution is added to each well. The plates are then incubated for 30 min at 37 °C and read automatically on a Titertek Fluoroscan II (Flow, Meckenheim, Germany).

EVALUATION

The results are given as absolute fluorescence units using 355-nm excitation and 460-nm emission filters. Statistical significance of the differences between the means is assessed by Student's *t*-test.

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P.10
Skin mycosis

P.10.1
General considerations

PURPOSE AND RATIONALE

Fungal infections of the skin account for a large number of consultations to general practitioners and dermatologists. They are caused by dermatophytes, such as Trichophyton rubrum, Trichophyton tonsurans, Trichophyton mentagrophytes, Microsporum canis, Epidermophyton floccosum, Microsporum gypseum, and Trichophyton verrucosum. (Sinski and Kelley 1991).

Most fungal skin infections, such as tinea pedis and tinea cruris, respond to topical therapy, although widespread or chronic infections that do not respond to local measures may require systemic treatment, for example with griseofulvin, ketoconazole or terbinafine. Traditional topical products such as compound benzoic acid ointment (Whitfield's ointment) and tolnaftate preparations have largely been superseded by other topical-active antifungal agents, such as the imidazoles (e.g. clotrimazole, miconazole or econazole) or hydroxypyridones (e.g. ciclopirox), which are well tolerated and rapidly effective (Gupta et al. 1994). Fungal infections also affect the appendices, such as hair and nails. To evaluate antifungal agents not only the antimicrobial spectrum, but also the penetration plays a decisive role.

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P.10.2
In vitro inhibitory activity

PURPOSE AND RATIONALE

In vitro tests are performed to investigate whether the test compound in comparison to standards covers the most relevant pathogens of dermal mycoses. The test conditions are rendered more difficult by addition of protein since the main infection site for fungi is the horny layer of the epidermis, which has a high protein content.

PROCEDURE

The studies are performed by means of conventional serial dilution procedures in test medium without and with addition of 4% bovine albumin. The test medium is Sabouraud dextrose broth containing 1% Neopeptone Difco (Difco Laboratories, Detroit, Mich., USA) and 2% glucose. The basic medium is sterilized in an autoclave at 121 °C for 15 min. The pH is adjusted to 6.5 with 1 N NaOH. The medium containing albumin is sterilized by filtration through a membrane filter. For preparation of the test series, the inhibiting substances are dissolved in methanol and then rapidly diluted with slightly warmed test medium, so that series with a continuous dilution factor of 2 are obtained: 125–0.03 µg/ml in medium without protein, 500–1 µg/ml in medium with protein. Each test tube contains 3 ml. The test organisms are various strains of dermatophytes (*Trichophytum mentagrophytes*, *T. rubrum*, *T. verrucosum*, *T. equinum*, *T. gallinum*, *Microsporum canis*, *Microsporum gypseum*) and yeasts (*Candida albicans*, *Ca. tropicalis*, *Ca. pseudotropicalis*, *Ca. krusei*, *Ca. parapsilosis*, *Ca. lipolytica*, *Ca. brumpti*, *Ca. utilis*, *Torulopsis glabrata*). The organisms are pre-cultured on a modified Grütz agar at 28 °C for periods of 1–4 weeks. The suspensions are adjusted by photometry that about 10^5 microconidia of dermatophytes and 10^4 yeast cells per ml are obtained in each inoculated test tube. The minimal inhibitory concentrations are measured after 14 days incubation at 28 °C.

EVALUATION

The percentage of strains of *Trichophytum mentagrophytes*, *Trichophytum rubrum*, and *Candida albicans* is plotted against dilution steps for each test compound with and without albumin and IC_{50} values are calculated.

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P.10.3
In vivo activity in the guinea pig trichophytosis model

PURPOSE AND RATIONALE

The guinea pig trichophytosis model has been used by several authors to evaluate antimycotic compounds (Millberger and Gillert 1973; Dittmar et al. 1981; Plempel et al. 1983; Petranyi et al. 1987; Schaude et al. 1990; Garcia Rafanell et al. 1992; Arika et al. 1993).

PROCEDURE

Male albino guinea pigs (Pirbright White), bred mycosis-free, weighing 450–550 g are fed Altromin® pellets and tap water ad libitum. On both sides of the back, areas of 5 × 12 cm are shorn to a fur length of 1 mm.

Three areas with a diameter of 3 mm are inoculated with a pipette on either side. Per injection site, 10^4 spores of *Trichophyton mentagrophytes* 2 114 in 0.05 ml suspension in physiological saline solution are inoculated. Three days after inoculation, infections with redding and scale formations are observed. From days 3–7 after the infection, 1 ml of the test preparation or standard is applied onto the right animal sides and rubbed in once daily. The diameters (mm) of all alopecias are measured with a ruler 3.5 weeks after the infection.

EVALUATION

The values of alopecias, separated according to the treated group and animal side, are determined and statistically evaluated using Duncan's new multiple range test.

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P.10.4 Skin penetration

PURPOSE AND RATIONALE

Cutaneous pathogenic fungi like dermatophytes or Candida ssp. present a particular affinity for the stratum corneum. Therefore the bioavailability of antimycotic drugs in the thick horny layer of the skin is an important element of treatment success. These studies can be performed in human cadaver skin (Dittmar 1981; Kligmann et al. 1987; Hänel et al. 1988)

PROCEDURE

Skin pieces, measuring 10×12 cm are removed from the back of corpses not later than 24 h after death. The subcutis is removed and the skin piece divided 3 to 5 parts. The edges (4 mm) of each individual piece are then taped with Tesa-film (adhesive tape) and the remaining surface is treated with the antimycotic compound. 0.05 ml of compound preparation or solution is rubbed into the skin for 10 s. For a period of one h, the pieces are placed on water agar, allowing for free admission of air. After this time, the compound is carefully wiped off with filter paper and water.

The skin area treated with the antimycotic is then stripped with Tesa-film tape as follows: (A) one third of the area remains unstripped; (B) one third of the area is stripped three times; (C) one third of the area is stripped six times. Then each piece of skin is placed into a Petri dish, which is heated in a water bath to 52 °C for 15 min. The epidermis is removed from all the skin pieces, which are then placed on slides (with the base turned upwards) and put into the upper parts of plastic Petri dishes. Each of the epidermis pieces is inoculated in 20 sites with a microconidia suspension of the dermatophyte Trichophyton mentagrophytes 109-FHM 1a. The spores had been rinsed off from three-week-old slant cultures, using distilled water. Approximately 50–100 viable spores are deposited at each inoculation site.

The upper parts of the Petri dishes (containing the slides with the epidermis pieces) are then covered with the bottom parts of Petri dishes, which contain 2% aqueous agar for producing a wet chamber. Holes in the covers serve to avoid excess humidity in the chamber. The chambers are incubated at 28 °C for 5 days. The fungal growth is recorded daily according to a three points score system.

EVALUATION

Growth is evaluated by visual scoring from days 2–5 after inoculation and calculation of AUCs from growth curves.

Inhibition values (%) are calculated according to the formula:

$$\text{inhibition} = \frac{\text{growth on controls} - \text{growth after treatment}}{\text{growth on contols}} \times 100$$

MODIFICATIONS OF THE METHOD

Ceschin-Roques et al. (1991) performed penetration studies on excised skin from the back of slaughtered pigs.

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P.11
Biomechanics of skin

P.11.1
General considerations

Several attempts were made to describe the mechanical properties of skin by mathematical models (Ridge and Wright 1965; Harkness 1971; Hirsch and Sonnerup 1968; Jamison et al. 1968; Viidik 1968, 1969, 1973, 1979; Frisén et al. 1969a,b; Veronda and Westman 1970; Danielson 1973; Soong and Huang 1973; Wilkes et al. 1973; Jenkins and Little 1974; Lanir and Fung 1974; Vogel 1976, 1986; Barbanel and Evans 1981; Barbanel et al. 1978; Lanir 1979; Barbanel and Payne 1980; Burlin 1980, 1981; Fung 1981; Sanjeevi 1982; Potts and Breuer 1983).

Most of these authors used models derived from studies in polymers (Ferry 1970). The simplest mechanical model analogous to a viscoelastic system is a spring combined with a dashpot, either in series (**Maxwell element**) or in parallel (**Voigt or Kelvin element**). Combinations of these elements were used to explain the mechanical phenomena in connective tissue, such as stress-strain behavior, relaxation and mechanical recovery, hysteresis and creep phenomena (Jamison et al. 1968; Frisén et al. 1969a,b; Hirsch and Sonnerup 1968; Vogel 1976, 1993; Riedl and Nemetscheck 1977; Vogel and Hilgner 1979; Viidik 1968, 1969, 1973, 1977, 1979). Larrabee (1986), Larrabee and Sutton (1986).

Larrabee and Galt (1986) reviewed the theoretical and experimental mechanics of skin and soft tissue and proposed a mathematical model of skin deformation based on the finite element method. A finite element based method to determine the properties of planar soft tissue was also described by Flynn et al. (1998).

Unfortunately none of these models has been found to be sufficient to describe all properties of human and animal skin including the mechanical history before measurement and the time-dependence during measurement. There is no comprehensive and unequivocally accepted model to describe completely the biorheology of skin. Therefore, several methods are used in order to get insight into the physical properties of skin.

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P.11.2 *In vitro* (*ex vivo*) experiments

P.11.2.1 Stress-strain behavior

P.11.2.1.1 Measurement of skin thickness, ultimate load, tensile strength, ultimate strain and modulus of elasticity

PURPOSE AND RATIONALE

Animal experiments are preferable when studying the biomechanical properties of the dermis, since only in animals can the values at higher extension degrees be studied, *ex vivo* or *in vivo* under anesthesia, whereas studies in humans are limited by pain threshold or to tests in cadaver skin. Skin thickness, ultimate load, tensile strength, ultimate strain and ultimate modulus of elasticity are the most informative parameters, which describe the mechanical properties of the dermis.

PROCEDURE

Groups of at least 10 male Sprague-Dawley rats with an initial weight of 120 ±5 g are treated subcutaneously or orally with test drugs or saline. The duration of treatment is usually 5 days; however, for special studies treatment can be prolonged up to 3 months. The animals are sacrificed under anesthesia. The back skin is shaved and a flap of 5×5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness. In this way, **skin thickness** can be measured reliably by calipers with an accuracy of 0.1 mm. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out (Vogel 1969, 1970, 1989, 1993a). The samples are kept in Peteri dishes until testing at room temperature on filter paper soaked with saline solution. The specimens are fixed between the clamps of an INSTRON® instrument a at gauge length of 30 mm. All measurements are carried out within at least 1 h. For long lasting test procedures, such as relaxation or cyclic loading, the samples are wrapped with saline soaked filter paper (Vogel 1976a,b 1989, 1993a,b).

Stress-strain curves are registered at an extension rate of 5 cm/min, showing a characteristic shape. During low strain values, there is a gradual increase of load; the curve has a concave part. The stress-strain curve ascends according to an exponential function (Vogel and Hilgner 1977). Afterwards an almost straight part is reached indicating the dependence on Hook's law. At this part the **ultimate modulus of elasticity** (Young's modulus) can be calculated (increase of load divided by the cross sectional area). Then some yielding of the curve occurs which ends in a sudden break of the specimen. This point indicates **ultimate strain** and **ultimate load**.

EVALUATION

From ultimate load divided by the cross sectional area (specimen width times original skin thickness measured at the beginning of the experiment), **tensile strength** can be calculated. The mean values of skin thickness, ultimate strain, ultimate load, tensile strength, and modulus of elasticity of treated animals are compared with controls using ANOVA and Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The parameters: **skin thickness, ultimate strain, ultimate load, tensile strength, and modulus of elasticity** are influenced by many factors, such as hormones and desmotropic drugs. The most pronounced changes are seen after glucocorticosteroids, both after systemic and local application (Vogel 1969, 1970, 1974a). Sev-

eral studies were performed on age dependence of mechanical parameters in rat skin. Ultimate load, tensile strength and modulus of elasticity show a very sharp increase during puberty, a maximum at 12 months and a slight decrease thereafter (Vogel et al. 1970; Vogel 1976b, 1978, 1983, 1988, 1989, 1993a). Studies on age dependence show a similar pattern for skin thickness, ultimate load, tensile strength, ultimate extension, modulus of elasticity, hysteresis, relaxation, creep behavior and biochemical data both in animals and men (Holzmann et al. 1971; Vogel 1987a,b).

Therefore, extrapolations from animal studies to behavior of human skin are justified.

However, due to the anatomical conditions (haired skin in animals) the biomechanics of the epidermis can be studied better in human experiments than in animals.

MODIFICATIONS OF THE METHOD

Measurement of skin thickness, ultimate strain, ultimate load, tensile strength, and modulus of elasticity can be used in the evaluation of topical corticosteroids (Schröder et al. 1974; Alpermann et al. 1982; Vogel and Petri 1985).

Similarly, Töpert et al. (1990) measured skin atrophy and tensile strength of skin after 30 days local administration of corticosteroids in rats.

Oxlund and Manthorpe (1982) found an increase of strength and a decrease of extensibility of skin strips after long-term glucocorticoid treatment of rats. In agreement with studies by Vogel (1974b, 1978), these results were explained by a change in collagen cross-linking pattern.

Jørgensen et al. (1989) found a dose-dependent increase of mechanical strength in intact rat skin after treatment with biosynthetic human growth hormone.

Andreassen et al. (1981) studied the biomechanical properties of skin in rats with streptozotocin-induced diabetes. An increased stiffness and strength was found: maximal stiffness was increased by 20% and the strain rate at maximum stress was decreased by 10%.

Oxlund et al. (1980) found that the stiffness of rat skin was increased in the early postpartum period. This increase was also found in adrenalectomized animals.

Among desmotropic drugs, l*athyrogenic* compounds, such as amino-acetonitrile, and *D-penicillamine* decrease ultimate load, tensile strength without major influence on skin thickness (Vogel 1971a,b, 1974a). A decrease of the strength of skin strips in rats after treatment with D-penicillamine was also found by Oxlund et al. (1984).

A dose-dependent increase of tensile strength in skin after treatment with was found with **non-steroidal anti-inflammatory drugs** (Vogel 1977).

Strain rate influences the values of ultimate load, tensile strength and modulus of elasticity, but not the effect of age and of corticosteroids (Vogel 1972b). Changes in tensile strength are correlated with the content of insoluble collagen (Vogel 1974b).

Fry et al. (1964) prepared skin rings from the lower part of the leg in rats and studied the age-dependence of the mechanical properties.

Nimni et al. (1966) measured tensile strength of excised skin samples in rabbits during aging.

Pan et al. (1998) studied ultrasound, viscoelastic and mechanical properties in rabbit skin, including stress relaxation, creep and Young's modulus as a function of strain.

Lofstrom et al. (1973) described circadian variations of tensile strength in the skin of two inbred strains of mice.

The effect of radiation therapy on mechanical properties of skin was studied in mice by Hutton et al. (1977) and by Spittle et al. (1980).

Schneider et al. (1988) measured tensiometric properties in guinea pig skin from flaps of normal dorsal skin and after implantation of an ovoid **tissue expander** filled for four days with saline.

Belkoff et al. (1995) studied the mechanical properties of skin in pigs after subcutaneous implantation and inflation of silicone tissue expanders.

Mustoe et al. (1989) compared the effects of a conventional tissue expansion regimen of 6 weeks with an accelerated regimen of 2 weeks in a model in dogs and measured skin thickness, elasticity, creep and stress relaxation.

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P.11.2.1.2 Measurement of mechanical properties at low extension degrees

PURPOSE AND RATIONALE

At low strain values, stress-strain curves of skin samples show a gradual increase of load, the curve has a concave part. The stress-strain curve ascends according to an exponential function (Vogel and Hilgner 1977).

PROCEDURE

Groups of at least 10 male Sprague-Dawley rats with an initial weight of 120 ±5 g are treated subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia; the back skin is shaved

and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out (Vogel 1969, 1970, 1981, 1989, 1993). The specimens are fixed between the clamps of an INSTRON® instrument at a gauge length of 30 mm. An extension rate of 5 cm/min is chosen. Tension is registered at 2, 5, 10, 20, 30, 40, 50, 60, 70, 80 and eventually 90% elongation. The registration is started with high sensitivity and switched to one tenth of the sensitivity in order to register the steep end of the curve.

EVALUATION

During registration with high sensitivity, the curve reaches an almost straight part. At this part, a tangent is drawn which is used for calculation of E_2. The distance from the start to the cross-point of this tangent with the baseline is measured and denominated as extension until the first rise (D). The angle between the tangent and the baseline is halved. At the cross-point with the stress-strain curve another tangent is drawn and used for calculation of E_1. At the second part of the curve registered with low sensitivity a further tangent is drawn for calculation of E_3, which is identical to the ultimate modulus of elasticity.

Mean values for $\boldsymbol{E_1}$, $\boldsymbol{E_2}$, $\boldsymbol{E_3}$, and ultimate stress of skin strips from treated animals are compared with controls using Student's *t*-test.

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P.11.2.1.3 Step phenomenon

PURPOSE AND RATIONALE

Analysis of the low parts of the stress-strain curve revealed a "step"-phenomenon (Vogel and Hilgner 1977, 1979a,b; Vogel 1988). If samples obtained perpendicular to the body axis are extended, a gradual increase of load is observed at low degrees of extension, which is suddenly interrupted by a decrease of the registered curve. Then the curve increases again, being interrupted by a second or third step.

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ±10 g are treated subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out. The ends of the skin strips are fixed in the clamps of an INSTRON® instrument resulting in a gauge length of 30 mm. The specimen is stretched with a strain rate of 50 mm/min. The first part of the stress-strain curve is registered with 10 times higher amplification than the second part. The steps are evaluated as follows.

EVALUATION

Tangents to the curves are drawn before and after each step. Then a vertical line is drawn in the middle of the step. The distance between the cross-points of this line with the tangents is measured and calculated as stress loss (ΔS). Furthermore, in the middle of each vertical line a horizontal line is drawn. The distance between the cross-point with the curves is measured and calculated as elongation due to the step (ΔE). Total stress loss is calculated by adding all values of ΔS and dividing by the number of specimens.

If this parameter is considered not only by itself but also in connection with tensile strength it can be calculated as percentage of ultimate stress (= % of breaking strength). In addition, the stress value, which would have been achieved without the step (= value S) and the elongation at which the step occurred (= value E) is registered. If stress loss at one step is multiplied with the elongation at this point, an indication of work loss is achieved (value ΔS times E). Taking into account the corresponding parameters indicating total work input (ultimate stress times ultimate strain), the relative work loss can be calculated.

Furthermore, elongation gain due to the steps is calculated by adding all values of ΔE and by dividing by the number of specimens.

Mean values of these parameters from treated animals are compared with controls using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The step phenomenon can be explained by the different orientation of collagen fibers in the dermis and by the presence of a muscular layer in rat skin. The muscular fibers are in a direction longitudinal to the body axis. If samples are obtained perpendicular to the body axis, the muscle bundles are cut transversally. In further studies the muscle layer was removed in one specimen and compared with a control still having the muscle layer. Investigation of the directional variation showed that the step phenomenon is mainly due to the muscular layer oriented longitudinal to the body axis and the connective tissue between the muscle bundles, whereas the anisotropic behavior of extensibility and ultimate strain is caused by the directional variation of the collagenous bundles in the dermis.

MODIFICATIONS OF THE METHOD

The step phenomenon could also be found in creep experiments (Vogel and Hilgner 1979b).

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P.11.2.1.4 Anisotropy of skin

PURPOSE AND RATIONALE

As is the case with human skin indicated by Langer's lines (Langer 1861; Gibson et al. 1969; Wright 1971; Stark et al. 1977; Daly 1982), the skin of rats exhibits **directional differences** (Hussein 1972, 1973; Vogel and Hilgner 1979a,b; Vogel 1981, 1983a, 1985a,b, 1988; Belkoff and Haut 1991). Stress-strain curves of rat skin showed a different shape if excised perpendicularly or longitudinally to the body axis. Directional variations of mechanical parameters in rat skin were studied depending on maturation and age (Vogel 1981).

PROCEDURE

Male Sprague-Dawley rats are used at different age groups from 1 week up to 24 months. Young animals (1, 2 and 3 weeks) are delivered with their mothers from the breeder. Each age group is randomly divided into 2 blocks which are assigned as "perpendicular to body axis" or "longitudinal to body axis". The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5×5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material of known thickness to measure skin thickness by calipers. From each rat two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out either perpendicular or longitudinal to the body axis, allowing a gauge length between the clamps of an INSTRON® instrument of 30 mm. Stress-strain curves are measured at a strain rate of 50 mm/min, whereby the first part of the curve is registered with 10-fold amplification. Besides ultimate values, the extension is measured at given load interval for each curve:

0.05, 0.1, 0.2, 0.5, 1, 2, 10, 20, and 50 N

and at given stresses:

0.01, 0.02, 0.05, 0.1, 0.5, 1, 2, 5, and 10 N/cm^2.

EVALUATION

Each parameter is measured longitudinal and perpendicular to the body axis. The mean values are statistically compared within each age group using Student's *t*-test.

At low loads, extension is higher in the longitudinal than in the perpendicular direction. The situation is reversed at medium and high stress values. Ultimate extension shows remarkable differences between longitudinal and perpendicular samples. Specimens obtained perpendicular to the body axis showed an increase during maturation, a maximum at 4 months of age and a decrease during further aging. The behavior of samples obtained longitudinal to the body axis was quite different. After an initial rise a maximum was found at 3 weeks. Afterwards a slight decrease was noted. Between 1 and 4 weeks all values of ultimate extension were significantly higher in samples punched out longitudinally to the body axis; between 4 and 12 months they were considerably lower (Vogel 1981).

CRITICAL ASSESSMENT OF THE METHOD

The data indicate the importance of directional variations in all studies of biomechanics of skin.

MODIFICATIONS OF THE METHOD

Directional variations of rat skin were also found in hysteresis experiments (Vogel 1983a) and in relaxation experiments (Vogel 1985a).

Directional variations of the stress-strain curves were also described in the skin of tight-skin mutant mice (Menton et al. 1978).

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P.11.2.1.5 Relaxation phenomenon

PURPOSE AND RATIONALE

In the relaxation experiment the viscous properties of rat skin are measured (Vogel 1973, 1976a,b, 1983, 1985, 1993a,b).

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ±10 g are treated subcutaneously or orally with test drugs or saline. The animals are sacrificed in anesthesia, the back skin is shaved and a flap of 5×5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

Skin strips are fastened between the clamps of an INSTRON® instrument and extended with the high strain rate of 1 000 mm/min up to 20% extension. This extension is kept constant for 5 min. The chart speed is initially 1 000 mm/min, then 10 mm/min. In this way, the **initial tension** and the **stress values at 0.001, 0.01, 0.1, 1, and 5 min** can be measured. Due to relaxation, the stress values drop down roughly with the logarithm of time.

Furthermore, the **residual stress** after 5 min relaxation period is measured and calculated as percentage of the original stress. After 5 min the sample is returned to 90% of the original strain, for example, from 20% to 18%. The stress following such unloading is recorded and again calculated as percentage of the original stress. Immediately after unloading, the measured stress values rise again spontaneously, which is called **mechanical recovery**. Mechanical recovery is calculated as percentage of initial tension and as percentage of stress after unloading. The relaxation experiment is repeated with increasing degrees of extension of 40%, 60%, 80% and eventually 100% until the specimen breaks.

EVALUATION

For each sample, the relaxation is calculated according to the formula

$$\sigma(t) = A_1 + A_{2\times} \log t$$

resulting in two constants (A_1 and A_2) for each sample whereby A_1 is the stress at $t = 0$, and A_2 the slope of the relaxation curve.

The **ratio between the constants A_1 and A_2** has to be considered as the most characteristic parameter of the relaxation experiment.

The means of these parameters are compared between treated animals and controls using Student's *t*-test.

MODIFICATIONS OF THE METHOD

In studies of age dependence in rats skin, a definitive decrease of the **ratio A_2/A_1** was found at 40% and 60%

extension degrees indicating a decrease of plasticity with age. Mechanical recovery, as an indicator of secondary elasticity, was better in old animals at medium extension degrees than in young individuals. Stress relaxation was decreased after corticosteroids and increased after thyroid hormones and D-penicillamine (Vogel 1973, 1993a,b).

Purslow et al. (1998) suggested that relaxation processes within the collagen fibers or at the fiber-matrix interface might be responsible for the viscoelastic behavior of skin.

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P.11.2.1.6 Hysteresis experiment

PURPOSE AND RATIONALE

In the hysteresis experiment not only the elastic but also the viscous properties of skin are measured (Vogel 1978, 1983).

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ±10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5×5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material of known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

Using the INSTRON® instrument, the samples are stretched up to a given extension degree (e.g., 20%) with an extension rate of 20 mm/min. When the given extension is achieved the crosshead is immediately moved back to the starting position with the same velocity. From the upward curve the stress and the modulus of elasticity at the end of the loading phase at the given strain indicating the elastic properties can be measured. When the sample is unstretched the unloading curve shows a different pattern reaching the baseline much earlier than the curve left it during the upward phase. From this point the **residual extension** can be measured. Immediately after the first hysteresis cycle, the experiment is repeated up to an extension degree of 30%, than to 40% and 50% and finally up to 60%.

EVALUATION

By planimetry of the area below the upward curve the **energy input** and of the area between the hysteresis loop the **energy dissipation** can be calculated as well as the **ratio between energy dissipation and energy input** at each hysteresis cycle indicating the viscous properties. Stress and modulus of elasticity at the end of the hysteresis loop, energy input, energy dissipation and the ratio between energy dissipation and energy input at each hysteresis cycle are compared between treated animals and controls using Student's *t*-test.

MODIFICATIONS OF THE METHOD

In rat skin, a maximum of the ratio between energy dissipation and energy input was found at 30% to 40% extension. This ratio is influenced by age. At low extension degrees there was an increase with age, whereas at high extension degrees an age-dependent decrease was noted. The ratio dissipation/input was slightly decreased by prednisolone, but definitively increased by D-penicillamine (Vogel 1993b).

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P.11.2.1.7 Isorheological point

PURPOSE AND RATIONALE

Buss et al. (1976) demonstrated the determination of the isorheological point to be a valuable parameter for the mechanics of connective tissue. This method has been modified and elaborated for the skin strips of rats (Vogel 1984, 1985, 1987).

PROCEDURE

Groups of 20 male Sprague-Dawley rats with an initial weight of 120 ±10 g are treated subcutaneously or orally with test drugs or saline. The groups are divided randomly into groups of 10 animals for examination of skin samples either perpendicular or longitudinal to the body axis. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5×5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

Being fastened between the clamps of an INSTRON® instrument, the specimen is expanded rapidly up to 2 N and the corresponding strain is measured. Keeping this strain constant, the load decay (relaxation) is measured for 5 min. Then the sample is again loaded up to 2 N and a second relaxation period of 5 min is evaluated. In the third cycle, the sample is unloaded to 50% of the load observed after the 5-min relaxation period in the second cycle. The phenomenon of **mechanical recovery** is observed. With the crosshead driven up and down, the point is sought where neither immediate relaxation nor mechanical recovery can be observed. The load and strain at this point define the **isorheological point**, which is characterized by the fact that under isometric conditions, the measured load is constant for several minutes. Increasing and decreasing the load by 10% produces a saw-tooth-shaped curve from which the **modulus of elasticity at the isorheological point** can be calculated. The same procedure is performed at higher initial loads such as 10 N and 50 N. The product of percentage of strain multiplied by stress at the isorheological point indicates energy density.

EVALUATION

The means of these parameters are compared between treated animals and controls using Student's *t*-test.

The values showed a decrease during maturation, a minimum at 12 months and an increase during senescence. In studies with desmotropic compounds, the decreased viscosity after treatment with prednisolone acetate was more evident at the isorheological points than at the ultimate values. Likewise, the higher extensibility after treatment with D-penicillamine was indicated more clearly by the isorheological points than by the ultimate strain.

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P.11.2.1.8 Creep experiments

PURPOSE AND RATIONALE

In creep experiments, viscous behavior of skin is studied. The strain under constant load is also denominated as retardation behavior. (Vogel 1977, 1987).

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ±10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5×5 cm removed. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis three dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

In a special apparatus, skin specimens are suddenly loaded with 100, 200 or 500 g and the extension degree measured. An immediate extension occurs which is followed by a slow and almost continuous creep being measured as **ultimate extension rate**. Furthermore, **extension achieved after 1 h** is registered.

EVALUATION

The means of these parameters are compared between treated animals and controls using Student's *t*-test.

An age-dependent decrease of these parameters was found in rat and human specimens indicating a decrease of viscosity or plasticity with maturation and age. Ultimate extension rate was decreased by prednisolone and increased by D-penicillamine.

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P.11.2.1.9 Repeated strain

PURPOSE AND RATIONALE
By the method of using repeated strain, mainly the viscous properties of skin are measured (Vogel and Hilgner 1978; Vogel 1987).

PROCEDURE
Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ±10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5 × 5 cm removed. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

Skin specimens are fastened between the clamps of an INSTRON® instrument, extended with a strain rate of 100 mm/min up to 20% extension and immediately unloaded followed by further cycles with the same strain rate and extension degree. The peak of the second cycle is considerably lower than the first, followed by a further decrease in the next cycles. The number of cycles is counted until the stress value is only one half of that of the first cycle. Immediately afterwards, the degree of extension is increased to 30%. Again the number of cycles is counted until the stress value was only one half of that of the first cycle. In this way, the **number of cycles indicating the half-life of tension** due to relaxation is counted at each step of 20%, 30%, 40%, 50%, 50%, 70%, 80%, 90% and eventually 100% extension. The number of cycles decreased from the first step (20%) to the third step (40%) and increased continuously until the last step. This increase was almost an exponential function of the number of steps.

EVALUATION
The means of the number of cycles indicating the half-life of tension due to relaxation at each step are compared between treated animals and controls using Student's *t*-test.

The parameters measured with this method are influenced by several factors: An increase was noted from an age of 1 month up to 24 months. The values were decreased by D-penicillamine and increased by prednisolone treatment. Again this method showed that plasticity of skin is decreased by age and by corticosteroids and increased by D-penicillamine.

MODIFICATIONS OF THE METHOD
Lafrance et al. (1998) tested mechanical properties of human skin equivalents submitted to cyclic forces. The *in vitro* production of disk-shaped (25.4 mm diameter) skin equivalents was based on the culture, under submerged conditions, of keratinocytes seeded on anchorage-based dermal equivalents, a human type I + III collagen gel supplemented with elastin and glycosaminoglycans. The specimens were submitted to quasi-static ramp-deflection cycles induced by means of an actuated hemispherical head. The effects of repeated loading were studied by monitoring the indentation load versus deflection and the relaxation of load over 1 000 s.

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P.11.2.2 Thermocontraction

PURPOSE AND RATIONALE
If collagenous material such as skin or tendon is heated in a water bath, thermocontraction occurs. This phenomenon was already described by Wöhlisch (Wöhlisch and du Mesnil de Rochemont 1927; Wöhlisch 1932). Verzár and other authors used the phenomenon of thermocontraction of tendons and skin strips extensively to study the ageing process (Verzár 1955, 1957; Lerch 1951; Rasmussen et al. 1964; Boros-Farkas and Everitt 1967; Viidik 1969, 1977, 1979; Vogel 1969). With increasing temperature, a sudden increase of isometric force is found, which is followed by a decrease. To evaluate this phenomenon, either the **shrinkage temperature** or the **stress at and above the shrinkage temperature** can be measured. Furthermore, the decrease of sample strength can be measured.

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ±10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5 × 5 cm removed. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

The samples are attached to the clamps of a specifically modified INSTRON® instrument which allows the submersion of the test specimen into a beaker filled with 0.9% saline solution. This solution is kept at 58 °C or 60 °C by a thermostat. Immediately after immersion, the exerted tension is measured and the **maximum of tension** and **time until maximal tension** are recorded.

EVALUATION

The means of these parameters are compared between treated animals and controls using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Joseph and Bose (1962) tested the shrinkage temperature of skin in newborn rats, at 10 months and 2 years and found an increase from 51.2 °C to 61.1 °C.

Alain et al. (1977, 1980) tested pieces of dorsal skin of rats. Temperature of maximum tension was decreased from birth to 1 month, and then very slowly increased with age. A rapid relaxation was observed in young rats and in non-senescent adult rats.

Rundgren (1976) found changes of thermal contractility in skin of young and old female rats, and also changes due to repeated pregnancies.

Blackett and Hall (1980) found an increase of thermal shrinkage temperature in two strains of mice during the aging period.

Danielsen (1981) determined thermal stability measured as area shrinkage without tension during heating for membranes of collagen fibrils, reconstituted from solutions of highly purified rat skin collagen.

Allain et al. (1980) built a device, which measured not only hydrothermal shrinking but also swelling in rat skin.

Le Lous et al. (1982a,b, 1983), Flandin et al. (1984) applied the technique of differential scanning calorimetry to evaluate the denaturation process of collagen in rat skin.

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P.11.3 *In vivo* experiments

P.11.3.1 Stress-strain curves *in vivo*

PURPOSE AND RATIONALE

In contrast to studies in human beings, animal experiments allow measurement of mechanical properties both *in vivo* under anesthesia and later on *in vitro* (*ex vivo*) at the same site. For this purpose, special methods had to be developed (Barbanel and Payne 1981; Vogel 1981a,b, 1982; Vogel and Denkel 1982, 1985; Denkel 1983).

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ±10 g are treated for 10 days subcutaneously or orally with test drugs or saline.

The rats are anesthetized with 60 mg/kg Nembutal® i.p. The back skin is shaved mechanically. Skin thickness is measured by use of calipers on an elevated skin fold. Four small (14 × 14 mm) metal plates bearing a hook are used as tabs and are glued on the skin in both longitudinal and perpendicular direction at a distance of 25 mm with a cyanoacrylate preparation. An operation table is mounted on the crosshead of an INSTRON® instrument. The table can be turned to allow stretching of the skin in both perpendicular and longitudinal directions relative to the body axis. The anesthetized animals are fastened by their legs to the operation table. A triangle is attached to the load cell. At the end of the triangle, threads are fastened which are conducted by reels and hooked to the tabs. The cross head is moved down manually until the threads are stretched, however, no tension is measured yet. Then the crosshead is driven downwards with a rate of 50 cm/min, what means that the actual extension rate is 100 cm/min. The load is measured only to limited values in order to prevent damage of the skin. In each rat the stress-strain curve is recorded in both directions whereby the order (first longitudinal or first perpendicular) is changed from animal to animal. In this way, the influence of the first extension on the results of the second extension is eliminated. Stress-strain curves are recorded up to an elongation of 80%. Modulus of elasticity is calculated from the almost straight part of the curve.

EVALUATION

Average stress-strain curves with standard deviations are plotted for each treatment group perpendicular and longitudinal to the body axis. The stress values and the values for modulus of elasticity of the treated groups are compared with controls using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

In spite of similar conditions, differences between the stress-strain curves from *in vitro* and *in vivo* experiments were found. These findings confirmed the statements of other authors (Barbanel and Payne 1981; Wijn 1980) of the importance of tab distance and tab geometry (Vogel 1981b). Analysis of the data showed that the higher the ratio of distance between the tabs to the area below the tabs, the higher were the stress values. These findings can be explained by the fact that skin consists of several layers. Only the upper layer (epidermis) is fastened to the tabs in the *in vivo* experiment. The forces transmitted to the lower layers are transmitted by a larger area if the area under the tabs is larger. The lower layer can slide over a larger area and is therefore less extended resulting in lower stress values. No sliding is possible if the sample is fastened from both sides as it is performed in the *in vitro* experiments. Taking into account all experimental conditions including the strain rate, the *in vivo* results are comparable with those obtained *in vitro*. This holds true for the age-dependence as well as for the influence of desmotropic compounds (Vogel and Denkel 1985).

MODIFICATIONS OF THE METHOD

Cook et al. (1977) compared tension/extension ratio curves *in vivo* and *in vitro* in rats using the suction-cup method. Baker et al. (1988) described an apparatus for testing mechanical properties of normal and irradiated **pig** skin *in vivo.* In this system, the pads were attached to the skin of the pig rump with double-sided tape. They were moved apart at a predetermined rate using a motorized unit. Force was assessed using a S-shaped, center point, double beam load cell mounted on a movable crosshead. Displacement of the pads was measured using a floating core linear variable displacement transducer. Using this system, Baker et al. (1989) studied the effect of single doses of X-rays on the mechanical properties of pig skin *in vivo*.

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P.11.3.2 Repeated strain *in vivo*

PURPOSE AND RATIONALE

A special method has been developed to study the mechanical properties of rat skin after repeated strain *in vivo* and the course of recovery during different time intervals (Denkel 1983; Vogel and Denkel 1985; Vogel 1988).

PROCEDURE

Male Sprague-Dawley rats with an initial weight of 120 ±10 g are treated for 10 days subcutaneously or orally with test drugs or saline. Groups of 10 animals are used for each time interval tested and for the two directions of testing.

Tabs are fastened on the shaved back skin of anesthetized rats with a distance of 25 mm either perpendicular or longitudinal to the body axis with a cyanoacrylate preparation. With an extension rate of 100 mm/min, the skin is extended 30 times for a 50% strain under anesthesia. Load is recorded and stress calculated by dividing load by skin thickness measured from a skin fold obtained with calipers. The 1st, 5th, 10th, 20th, 25th and 30th cycles are recorded with faster paper speed in order to facilitate the evaluation of modulus of elasticity and stress values. Modulus of elasticity is calculated from the upper part of the stress-strain curve. The area under the curve of 30 cycles is evaluated by computerized calculation according to Simpson's formula (Hütte 1915; Denkel 1983; Vogel 1988).

EVALUATION

Average curves of stress vs. number of cycles with standard deviations are plotted for each treatment group perpendicular and longitudinal to the body axis. The stress values and the values for modulus of elasticity of the treated groups are compared with controls using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

Stress values decreased after repeated loading approximately with the logarithm of number of cycles. Stress values and the area under the curve were higher perpendicular than longitudinal to the body axis, as found in other *in vivo* experiments (Vogel and Denkel 1985). During the experiment, modulus of elasticity increased in both directions from the first to the fifth cycle. This may be explained by the so-called "conditioning" of connective tissue (Nemetscheck et al. 1980). From the 5th to the 30th cycle, a decay of modulus of elasticity approximately with the logarithm of number of cycles was noted. The area under the curve calculated from stress values resembled closely the pattern of initial stress, indicating that this value dominates for the area under the curve and that the decay is only of secondary importance.

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P.11.3.3 *In vivo* recovery after repeated strain

PURPOSE AND RATIONALE

A method was developed to study the *in vivo* recovery of mechanical properties of rat skin after repeated strain (Denkel 1983; Vogel and Denkel 1985; Vogel 1988, 1990, 1993a,b). Full recovery, i.e., ***restitutio ad integrum***, can be observed only by doing *in vivo* experiments but not by *in vitro* experiments.

PROCEDURE

Male Sprague-Dawley rats with an initial weight of 120 ±10 g are treated for 10 days subcutaneously or orally with test drugs or saline. Groups of 10 animals are used for each time interval tested and for the two directions of testing.

As in the experiment of repeated strain, tabs are fastened on the shaved back skin of anesthetized rats with a distance of 25 mm either perpendicular or longitudinal to the body axis with a cyanoacrylate preparation. With an extension rate of 100 mm/min, the skin is extended 30 times for a 40% strain under anesthesia. Load is recorded and stress calculated by dividing load by skin thickness measured from a skin fold obtained with calipers. The 1st, 5th, and 30th cycles are recorded with faster paper speed in order to facilitate the evaluation of modulus of elasticity and stress values. Modulus of elasticity is calculated from the upper part of the stress-strain curve. The area under the curve of 30 cycles is evaluated by computerized calculation according to Simpson's formula (Hütte 1915; Denkel 1983; Vogel 1988).

After the first run the animals are returned to their cages with the tabs still in position. A second run of repeated strain is applied after different time intervals at 1, 6, and 16 h. The stress values in the second run are definitively lower than in the first run.

When calculated as percentage of the first run, the differences diminish with extended time intervals. By this *in vivo* method not only the mechanical recovery, which can also be observed *in vitro*, but also the biological recovery, i.e., the restitutio ad integrum, can be measured. Almost full recovery is found after 16 h.

EVALUATION

Average curves of stress vs. number of cycles with standard deviations are plotted for each treatment group perpendicular and longitudinal to the body axis. The stress values and the values for modulus of elasticity of the treated groups are compared with controls using Student's *t*-test. Stress of the first and 30th cycle as well as the area under the curve at the second run are expressed as percentage of the first run after the 1, 6, or 16 h interval. Furthermore, the hours to reach certain percent levels up to 100% are calculated for the stress values during the first cycle and the area under the curve both longitudinally and perpendicularly to the body axis.

CRITICAL ASSESSMENT OF THE METHOD

In the late phase of recovery, an even better restitutio ad integrum was found in animals treated with 300 mg/kg p.o. D-penicillamine or 10 mg/kg s.c. prednisolone acetate. In contrast to other biomechanical parameters, the restoration process was found to be barely influenced by treatment with desmotropic compounds.

Surprisingly, in studies on age dependence restitutio ad integrum was the fastened in old animals. The ability of the dermis to reconstitute the fibrous structure is apparently not influenced negatively by age.

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P.11.4 Healing of skin wounds

PURPOSE AND RATIONALE

Healing of skin wounds is a multiphasic process. The effect of drugs on the healing process was studied by measuring the mechanical strength at various time intervals after incision of the skin (Vogel 1970).

PROCEDURE

Groups of 10 to 20 male Sprague-Dawley rats weighing 120 ±5 g are used for each dosage or control and for each test interval. Under anesthesia the dorsal skin is shaved and an approximately 3 cm long incision is made down to the fascia in a cranio-caudal direction in the dorso-lumbar region. Immediately afterwards, the wound is closed with wound clips. The rats are treated subcutaneously with test drugs beginning on the day of surgery. The clips are removed the day before the tensile strength is tested or on the 10th postoperative day at the latest.

For the measurement of wound tensile strength, the rats are sacrificed under anesthesia on days 3, 6, 9, or 12 after surgery. Wound clips are fastened *in situ* on each side of the incision and connected by means of threads with the load cell and the crosshead of an INSTRON®-Instrument. Stress-strain curves are recorded at an extension rate of 5 cm/min. **Dehiscence of the wound** results in a sudden drop of the registered load. For experiments up to 3 weeks, bell-shaped skin strips are punched and tensile strength is tested, as described for evaluation of tensile strength in normal skin.

EVALUATION

Mean values of tensile strength of skin wounds in drug treated groups at each time interval are compared with controls using Student's *t*-test. To visualize the influence of drugs on the healing process, the changes following treatment with drugs are expressed as percentage of vehicle treated controls.

A dose-dependent decrease following treatment with corticosteroids is found after immediate postoperative treatment and a dose-dependent increase in prolonged experiments. This data serve as comparative parameters for new test compounds

MODIFICATIONS OF THE METHOD

Many authors measured tensile strength of skin wound to follow the course of wound healing under various conditions. Most experiments were performed in *rats* (Struck et al. 1967; Holm-Pedersen and Zederfeldt 1971; Holm-Pedersen and Viidik 1972a,b; Andreassen et al. 1977; Greenwald et al. 1993; Seyer-Hansen et al. 1993; Jyung et al. 1994; Adamson et al. 1996; Quirinia and Viidik 1998; Canturk et al. 1999; Gupta et al. 1999), with some of them also using the INSTRON®-Instrument (Phillips et al. 1993; Maxwell et al. 1998; Jimenez and Rampy 1999; Kim and Pomeranz 1999).

A biphasic effect of corticosteroids on wound healing in rats was also found by Oxlund et al. (1979).

Furthermore, **mice** (Butler et al. 1991; Celebi et al. 1994; Kashyap et al. 1995; Vegesna et al. 1995; Gonul et al. 1998; Matsuda et al. 1998), **guinea pigs** (Bernstein et al. 1991; Drucker et al. 1998; Silverstein and Landsman 1999), **rabbits** (Sandblom 1957; Wu and Mustoe 1995; Pandit et al. 1998, 1999; Knabl et al. 1999; Xia et al. 1999), **dogs** (Howes et al. 1929; Scardino et al. 1999), **pigs** (Langrana et al. 1983; Higashiyama et al. 1992; Chang et al. 1998; Fung et al. 1999) or **Yukatan miniature pigs** (Van Dorp et al. 1998) were used.

In some studies simultaneously polyvinyl alcohol sponges were implanted in which collagen accumulation was determined (Albina et al. 1993; Schaffer et al. 1996; Koshizuka et al. 1997; Bitar 1998; DaCosta et al. 1998; Witte et al. 1998).

Ågren and Mertz (1994) found excessive granulation tissue formation and retarded wound contraction in wounds in tight-skin mice.

Kyriakides et al. (1999) reported accelerated wound healing in mice with a disruption of the thrombospondin 2 gene.

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P.12
Protection against UV light

PURPOSE AND RATIONALE

The mechanical parameters' skin thickness, ultimate strain, ultimate load, tensile strength and modulus of elasticity were used to evaluate the effects of UV-irradiation and the prevention of skin damage in hairless mice (Alpermann and Vogel 1978; Vogel et al. 1981).

PROCEDURE

Female hairless mice (strain mutant h/h) with an initial weight of 20 ±2 g are used. Groups of 20 animals are treated topically with 0.025 ml of the sunscreen

product or the base. One other group is not treated topically, but irradiated. A further group is neither treated nor exposed to UV-B.

A special light source (Osram Ultra-Vitalux No. 2) is used. This light source is almost free from UV-C. For UV exposure groups of 5, mice are immobilized under a fine wire net of 14×14 cm at a distance of from the lamp, resulting in an irradiation energy of 14 mW/cm^2 for UV-B and 33 mW/cm^2 for UV-A. Irradiation is performed once a day except Saturday and Sunday during 4 weeks. Exposure time is 45 s in the first week, 60 s in the second, 90 s in the third, and 120 s in the fourth week. The animals are sacrificed umder anesthesia 72 h after the last irradiation. A flap of back skin is removed, skin thickness measured by use of calipers and two dumb-bell shaped samples punched with a width of 4 mm in the middle of the specimen. Stress-strain curves are recorded with an INSTRON®-instrument at a strain rate of 5 cm/min. Ultimate load and ultimate strain are recorded and tensile strength as well as ultimate modulus of elasticity are calculated.

Furthermore, samples of skin are deep-frozen for chemical analysis of collagen and soluble collagen fractions as well as of elastin (Vogel 1978).

EVALUATION

The mean data from animals treated with sunscreen products are compared with those of animals treated with ointment base, irradiated and non irradiated controls using ANOVA and Student's *t*-test.

MODIFICATIONS OF THE METHOD

The effect of UV-irradiation on skin has been studied by several authors both in man and in animals.

Wolska (1974) recommended the hairless mouse as an experimental model for evaluating the effectiveness of sunscreen preparations.

Cook et al. (1979) investigated the changes in the mechanical properties of intact **guinea pig** skin resulting from ultra-violet irradiation.

Lowe and Breeding (1986) evaluated several sunscreen preparations by UVB irradiation of mice. Sunscreen solutions were applied to the back of 5–8 weeks old female skh/HR-1 mice one h before irradiation with FS40 sunlamps. Epidermal DNA synthesis assay was used to measure sunscreen efficacy. The amount of UVB required to achieve a 50% suppression of radiolabelled thymidine incorporation in treated and untreated mice was compared as a ratio to determine the protective factor. Furthermore, epidermal ornithine decarboxylase activity was determined by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine 24 h after a single UVB exposure. Edema was estimated by the increase in double skin fold thickness measured by a caliper at 24 h post irradiation.

Benrath et al. (1995) reported that substance P and nitric oxide mediate wound healing of ultraviolet photodamaged skin in **rats**.

Muizzudim et al. (1998) studied the effect of topical application of antioxidants and free radical scavengers on protection of hairless mouse skin, exposed to suberythemal doses of ultraviolet B, three times a week, and measured epidermal thickness by microscopy.

Fullerton and Keiding (1997) quantified UV-B induced erythema in depilated Hartley-strain albino male guinea pigs and compared the results with a tristimulus colorimeter (Minolta ChromaMeter CR-200) and two spectrophotometers (Minolta Spectrophotometer CM-508i and CM-2 002). With the tristimulus colorimeter the color is expressed in a three-dimensional color space, which simulates the perception of color by the human eye.

Further description of ultraviolet erythema in animals see Sect. H. 3.2.2.1.

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P.13
Transepidermal water loss (TEWL)

PURPOSE AND RATIONALE

The physical basis for the measurement of transepidermal water loss (TEWL) is the diffusion law discovered by A. Fick in 1855.

$$dm / dt = -D \times A \times dp / dx$$

where:

- A = surface (m^2),
- m = water transported (g),
- t = time (h),
- D = diffusion constant (= 0.0877 g/m × h × mm Hg),
- p = vapor pressure of the atmosphere (mm Hg),
- x = distance from skin surface to point of measurement.

Most of the recent studies are performed with commercially available instruments, such as Tewameter TM 210, Courage and Khazaka, Cologne, Germany, Evaporimeter EP1, Servo Med AB, Vallingby, Sweden, primarily designed for the used in human beings.

Several studies were performed in **rats**, e.g., in hairless rats (Doucet et al. 1991; Vanbever et al. 1998), neonatal rats (Wickett et al. 1995), in rats with experimentally induced condition of essential fatty acid deficiency (Basnayake and Sinclair 1956; Prottey et al. 1976; Hartop et al. 1976, 1978; Penneys 1992; Yamaguchi et al. 1998; Meguro et al. 2000), **hairless mice** (Grubauer et al. 1989; Mortz et al. 1997; Sato et al. 1998), essential fatty acid deficient hairless mice (Menton 1968; Lowe and Stoughton 1977), platelet-type 12-lipoxygenase deficient mice (Johnson et al. 1999), keratin 10 deficient mice (Jensen et al. 2000), **guinea pigs** (Frosch et al. 1993; Fuchs et al. 1998; Sagiv et al. 2000), **pigs** (Zhao and Singh (1999) **Yucatan microswine** (Gendimenico et al. 1995).

Löffler et al. (2001) evaluated irritant skin reaction in mice by measurement of auricular transepithelial water loss.

PROCEDURE

BALB/c mice aged 10–16 weeks are sensitized on day 0 by applying 50 µl 2,4-dinitro-flurobenzene (DNFB) solution (0.5% diluted in acetone/olive oil 4 : 1) to the shaved dorsal neck region. On day 5, the dorsal surface of one ear is challenged by applying 10 µl DNFB 0.3%; the other side is treated by acetone/olive oil alone.

Transepithelial water loss is measured with an evaporimeter (Tewameter TM 210, Courage and Khazaka, Cologne, Germany) with a measuring cylinder into which the whole ear of the mouse can be placed. The measurements are performed under isofluoran inhalation anesthesia. Measurements are performed before and 24 h after challenge.

EVALUATION

The comparison between the treated and untreated ears is calculated by the Wilcoxon test. The comparison between the tested groups is calculated by the Kruskal-Wallis *H*-test, after the Mann-Whitney *U*-test.

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P.14
Influence on hair growth

PURPOSE AND RATIONALE

Several mutations affecting hair growth or quality have been reported in the laboratory mouse (Holland 1988), among them "alopecia" (Dicke 1955); "alopecia periodica" (Tutikawa 1952); "crinkled" (Falconer et al. 1951); "frizzy" (Falconer and Snell 1952); "fuzzy" (Mann 1964); "rhino" (Mann 1971); "naked" (Raphael et al. 1982); "nude" (Flenagen 1966; Buhl et al. 1990; Militzer 2001); "ragged" (Slee 1962); androchronogenetic alopecia (AGA) mouse (Matias et al. 1989); aging C3H/HeJ mice (Sundberg et al. 1994); SPF-ASH mice (Shimada et al. 1994); "nackt" (Benavides et al. 1998); transgenic mice overexpressing homeobox gene MSX-2 (Jiang et al. 1999). Kligman (1988) used the Skh-hairless mouse as a model for evaluating promoters of hair growth.

PROCEDURE

Six litters of Skh-hairless-1 albino mice with 5–8 animals are housed, with their mothers, in individual plastic cages. Food and water are supplied ad libitum. Beginning at 1 week of age, topical treatment is applied: two litters dorsal application of low dose (e.g., 0.2% minoxidil), two litters dorsal application of high dose, two litters abdominal application of high dose. Application is performed once daily five times a week at a rate of 20 µl/cm^2 of skin using a digital micropipette. Two control litters receive no treatment. All treatment continue for up to 17 weeks, after which mice are sacrificed and grossly examined for signs of toxicity.

Mice are observed daily for hair growth and weighed each week. Photographs are taken when hair growth appears to be maximum. At the time of maximal hair growth, two or three representative neonates from the treated and untreated groups are sacrificed for skin biopsies; all remaining mice continue on treatment to asses the extent of a third pelage. Biopsy specimens are fixed in formalin and stained with hematoxylin and eosin.

EVALUATION

In transverse sections, all active hair matrices or viable follicles, which contain keratin zed hairs located in the subcutis, are counted. Fourteen to 16 contiguous fields are examined over a surface distance of 1 cm at 250× magnification. Mean values of the groups are compared using Student's *t*-test.

MODIFICATIONS OF THE METHOD

A hair loss mutation on mouse chromosome 19, called **scraggly**, was described by Herron et al. (1999).

Several authors studied **chemotherapy-induced alopecia in mice, rats and rabbits** (Powis and Kooistra 1987; Paus et al. 1994a,b; Cece et al. 1996; Sredni et al. 1996).

The **Dundee experimental bald rat (DEBR)** was used as model for alopecia areata (Oliver and Lowe 1995; McElwee et al. 1997).

Kimura (1996) underlined the usefulness of studies in **hairless descendants of Mexican hairless dogs** in dermatological science.

The **balding stumptail macaque** is recommended as a model for androgenetic alopecia (Brigham et al. 1988; Diani et al. 1995; Pan et al. 1998).

The stimulating effect of drugs on hair growth has been studied using *in vitro methods.*

Kurata et al. (1996) investigated the effect of hypertrichotic agents on follicular from macaque and human skin and nonfollicular cells (normal kertainocyts and dermal fibroblasts) *in vitro*. Minoxidil induced a significant increase in all follicular cells in a dose-specific manner, whereas nonfollicular cells showed no response.

Lachgar et al. (1996) found inhibitory effects of bFGF, VEGF and minoxidil on collagen synthesis by cultured hair dermal papilla cells from rat vibrissa follicles.

Boyera et al. (1997) described biphasic effects of minoxidil on the proliferation and differentiation of normal human keratinocytes obtained form microdissected hairs of from plucked hairs. Minoxidil stimulated human keratinocyte proliferation at micromolar doses, while antiproliferative, pro-differentiative and partially cytotoxic effects were observed with millimolar concentrations.

Sato et al. (1999) reported that minoxidil increases 17â-hydroxysteroid dehydrogenase and 5á-reductase of cultured human dermal papilla cells from balding scalp.

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P.15
Cutaneous microcirculation

P.15.1
General considerations

PURPOSE AND RATIONALE

Various techniques are used to determine cutaneous blood flow, such as radioactive microspheres, xenon clearances, plethysmography, laser Doppler velocimetry (Flagrell 1986; Guy et al. 1985), reflectance spectrophotometry (Kimura et al. 1988; Kakizoe et al. 1992).

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Kakizoe E, Kobayashi Y, Shimoura K, Hattori K, Jidoi J (1992) Real-time measurement of microcirculation of skin by reflectance spectrophotometry. J Pharmacol Toxicol Meth 28:175–180
Kimura T, Tsushima N, Nakayama R, Yoshizaki S, Otsiji K, Shinohara M (1988) Effects of prostaglandin E_1 on the microcirculation of skin in the arteriosclerosis obliterans. J Jpn Coll Angiol 28:177–181

P.15.2
Laser Doppler velocimetry

PURPOSE AND RATIONALE

Laser Doppler velocimetry and laser Doppler flowmetry are essentially identical procedures. With these methods, light is transmitted from a helium-neon laser source in the instrument to the skin via an optical fiber. The laser provides light of single frequency (wavelength 632.9 nm) and allows the Doppler effect to be exploited. The incident radiation enters the skin tissue and is multiply scattered and reflected by non-moving components and by the mobile red blood cells that are encountered as the radiation penetrates to a depth of 1–1.5 mm. A portion of scattered/reflected incident radiation exits the skin and is collected by second or third optical fibers that carry the light back to the instrument. The returning radiation falls on a photodetector and is converted to an electrical signal. Stationary skin tissue reflects and backscatters light at the same frequency as the incident source. Erythrocytes moving with certain velocity, however, reflect radiation that is slightly frequency-shifted, the shift increasing with increasing velocity.

Hirkaler and Rosenberger (1989) described simultaneous two-probe laser Doppler velocimetric assessment of topically applied drugs as a simple, non-invasive method for the determination of cutaneous blood flow in anesthetized rats.

PROCEDURE

Male Sprague-Dawley rats (400–500 g) are anesthetized with urethane (1.6 g/kg), the abdominal region carefully clipped and the remaining hair removed with a commercially available depilatory. Phthalate buffer (pH 4.0) is gently applied to neutralize the effects of the depilatory cream. The rat is placed on its back and allowed to stabilize for approximately 15 min. Cutaneous blood flow is measured using two standard Medpacific LD 5 000 capillary perfusion monitors and probes (1.9 cm diameter). The probes are modified to allow application of the drug without removal of the probe. This is achieved by enlarging the center opening of the adhesive pad to 1 cm, which creates a drug well of approximately 5 µl vol. The drug is introduced through a short length of polyethylene tubing (PE10) held in place by the adhesive pad and placed slightly off center to avoid interference with the sensing device.

The modified probes are attached to the lower abdominal region of the rat, approximately 1–2 cm from the midline. Cutaneous blood flow is recorded continuously from 30 min prior to drug application and for 5 h post-dosing. Various doses of standard (0.015 to 0.75 mg/kg minoxidil) or test compound or control vehicle (propylene glycol 15%, ethyl alcohol 65%, water 20%) are applied. At the end of the experiment, the animals are sacrificed.

EVALUATION

Post-dosing values are expressed as % change from control and analyzed using Student's paired *t*-test.

MODIFICATIONS OF THE METHOD

Knight et al. (1987) measured microvascular blood flow by a laser Doppler flow meter in **rabbit** epigastric island flaps made ischemic for various intervals of time.

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Guy RH, Tur E, Maibach H (1985) Optical techniques for monitoring cutaneous microcirculation. Int J Dermatol 24:88–94
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Knight KR; Collopy PA, O'Brien BM (1987) Correlation of viability and laser Doppler flowmetry in ischemic flaps. J Surg Res 43:444–451

P.15.3 Measurement of skin microcirculation by reflectance spectroscopy

PURPOSE AND RATIONALE

Kimura et al. (1988), Kakizoe (1992) developed reflectance spectrophotometric measurement to analyze the microcirculation of the skin in real time. The light reflected from the skin tissue containing information about hemoglobin, the injected dye, and other data, is continuously analyzed into the relative absorption spectrum and the changes in relative absorption values at specific wavelengths are used as indices of oxyhemoglobin content and capillary permeation.

PROCEDURE

Male Wistar rats weighing 300–400 g are anesthetized with pentobarbital (40 mg/kg i.p.). The skin of the back is shaved and the hair removed by a commercial hair remover. A polyethylene catheter (PE10) is inserted into the femoral artery to measure blood pressure on a polygraph. An other catheter is inserted into the femoral vein for injection of drugs or Evans blue.

To obtain the relative absorption spectra, a spectro-multi-channel-photodetector system (MCPD 110; Otsuka Electronics Co., Ltd., Osaka, Japan) is used. White light (halogen lamp 150 W) is projected onto the skin through an optical fiber. The light reflected from the skin is transmitted to the detector system via another optical fiber. The positions of the optical fibers are fixed independently. Each fiber is attached to a steel arm on the stand, and the height of the arms is adjusted so that the top of the fibers can be in gentle contact with surface of the skin. The angles of the fibers are adjusted so that the relative absorbance spectrum with the best peaks of oxyhemoglobin can be obtained. The reflected light is passed through a slit and illuminated on the grating surface to obtain the spectrum. Following amplification by an image intensifier, the component wavelengths are sampled by photodiode array in a short time interval. The relative absorbance spectra from the skin against the spectra for the white light are obtained at wavelengths ranging from 450 to 643 nm, arranging the initial relative absorbance at 640 nm to zero. The sampling time is 50 ms. The average of 10 measurements is indicated at 1-s intervals on the output unit. The spectrum at a point of time and time-dependent changes in relative absorbance values at selected wavelength are shown on the display and registered by an X-Y recorder.

After achieving a steady state under anesthesia, the relative absorbance spectrum is obtained from the skin, and two peaks ate wavelength of about 540 and 577 nm, corresponding to those of oxyhemoglobin, are observed. The relative absorbance spectrum is measured at the posterior part of the back near the backbone because the shape is relatively flat in this region and it is easy to fix the optical fibers.

To standardize the changes in oxyhemoglobin content in skin tissue, graduated doses of noradrenaline are injected intravenously and the effect on relative absorbance is measured.

To measure the content of oxyhemoglobin and permeation of the capillaries at the same time, 1.5 ml/kg 0.05% Evans blue solution is injected intravenously 10 min before measurement. Histamine (0.3–100 μg/50 μl/site) is injected intradermally into the skin of the back, and measurement is started 1 min after the injection. The changes in the relative absorbance values at 540 nm, an absorption peak in the oxyhemoglobin spectrum, and at 610 nm, an absorption peak in the Evans blue spectrum, are measured for 15 min, and the absorption values at baseline and the point of maximum change are compared.

EVALUATION

Results are expressed as mean ±SEM. Comparisons are made using the Student's *t*-test or Mann-Whitney's *U*-test.

MODIFICATIONS OF THE METHOD

Hertel (1986, 1992) measured cutaneous microcirculation in the pinnal of conscious rats. Erythrocyte flow velocities were measured by the 'flying spot technique' (Tyml and Ellis 1982) and the diameters of the capillaries were measured from a monitor with a ruler.

Da Costa et al. (1992) measured the fluctuations in the diameter of selected arterioles in the cutaneous microcirculation of **Syrian golden hamster** dorsal skin flap chambers. These ranged in size between 10 and 70 μm at different branching order sites, before burn, at the same site after burn and after injection of drugs.

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Chapter Q
Guidelines for the care and use of laboratory animals

Q.1
Regulations for the care and use of laboratory animals in various countries[1,2]

Table Q.1. Regulations for the care and use of laboratory animals

Country, Institutions	Legislation
Australia National Health and Medical Research Council Commonwealth Scientific and Industrial Research Organisation Australian Agricultural Council	Australian Government Publishing Service (ed): "Australian code of practice for the care and use of animals for scientific purposes", Canberra 1991 *http://www.act.gov.au/environ/policy/sci.html*
Austria	Tierversuchsgesetz – TVG (Bundesgesetz vom 27. September 1989 über Versuche an lebenden Tieren) BGBl. Nr. 501/1989, zuletzt geändert durch BGBl. I Nr.169/1999 *http://www.bmwf.gv.at/4fte/forecht/tierversuch/tierversu.htm*

[1] With contributions to the first edition by GGF (Gesellschaft für Gesundheit und Forschung, Frankfurt am Main, Germany, revised for this edition by U. Albus.

[2] A second edition of a table of regulations on the use of animals in research has been published in 2001 by the European Science Foundation, Strasbourg.

Table Q.1. *Continued*

Country, Institutions	Legislation
Belgium	F.86 - 1896, 14 Août 1986, Loi relative à la protection et au bien-être des animaux (national law on animal welfare) F.94 - 28, 14 Novembre 1993, Arrêté royal relatif à la protection des animaux d'expérience (a Royal decree)
Canada	Canadian Council on Animal Care *http://www.ccac.ca/english/gublurb.htm*
China (Peoples Republic of China) State Science and Technology Commission, Beijing	Regulations for the Administration of Affairs Concerning Experimental Animals, Nov. 1988 Implementing Regulations of the Administration on Medical Experiments on Animals, June 1989
Denmark	LBK (Lovbekendtgørelse) nr. 726 af 9. sept. 1993: Bekendtgørelse om lov om dyreforsøg A minor change has been added to the above about animals that are released in connection to an experiment: • LOV nr. 386. 6 juni 1991 (animal protection law) • LOV nr. 1081 af 20. dec. 1995: Lov om ændring af lov om dyreforsøg • BEK (Bekendtgørelse) nr. 332 af 18. maj 1990: Bekendtgørelse om fremskaffelse af dyr til forsøg • BEK nr. 27 af 22. jan. 1996: Bekendtgørelse om ændring af bekendtgørelse om fremskaffelse af dyr til forsøg • BEK nr. 716 af 1. aug. 1994: Bekendtgørelse om forretningsorden for Rådet for Dyreforsøg • BEK nr. 333 af 18. maj 1990: Bekendtgørelse om forsøgdyrs pasning of opstaldning of om udryddelsestruede og vildtlevende dyr til forsøg m.v. • BEK nr. 739 af 6. dec. 1988: Bekendtgørelse over og indberetning om dyreforsøg Legislation revised in 1993 in conformity with EU directives *http://www.retsinfo.dk/*
European Union	Council of Minister's Directive 86/609/EEC (1986) on the Approximation of Laws, Regulations and Administrative Provisions of the Member States Regarding the Protection of Animals Used for Experimental and other Scientific Purposes *http://europa.eu.int/* European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. December 15, 1990 *http://www.uku.fi/laitokset/vkek/Sopimus/convention.html*
Federal Republic of Germany Ministry for Food, Agriculture and Forestry	Tierschutzgesetz vom 25. Mai 1998 Richtlinie vom 24. November 1986 zur Annäherung der Rechts- und Verwaltungsvorschriften der Mitgliedstaaten zum Schutz der für Versuche und andere wissenschaftliche Zwecke verwendeten Tiere (86/609/EWG) Gesetz zu dem Europäischen Übereinkommen vom 18. März 1986 zum Schutz der für Versuche und andere wissenschaftliche Zwecke verwendeten Wirbeltiere vom 11. Dezember 1990 *http://www.bml.de/* and *http://www.uni-giessen.de/tierschutz/*
Finland	Finnish Law on Animal Welfare Statutes "Laboratory animals and their use in scientific research", 1986 Legislation in Finland meets the EU demands *http://www.uku.fi/laitokset/vkek/english.html*
France	"Loi à la protection de la nature", 10 juillet 1976 "Décret aux expériences pratiquées sur les animaux" 19 octobre 1987 Arrêtés interministériels (3 arrêtés), 19 avril 1988 *http://www.cnrs.fr./SDV/expanim.html*
Italy	Legislative Decree no. 116 of January 27, 1992, enforcing European Recommendations contained in Directive 86/609/EEC Specific Law of October 12, 1993
Ireland Minister for Health and Children	Cruelty to Animals Act, 1876 European Communities (Amendment of Cruelty to Animals Act, 1876) Regulations, 1994 *http://www.doh.ie/policy/animal/*
Japan Prime Minister authorized to set standards for minimum pain provision	Law Concerning the Protection and Control of Animals (Law No. 105, October 1, 1973) *http://hayato.med.osaka-u.ac.jp/index/guide/inform/regulation/law1.html*
New Zealand Animal Welfare Advisory Committee (AWAC), established in 1989 by the Minister of Agriculture	Animal Welfare act 1999, Commenced January 1, 2000 *http://rangi.knowledge-basket.co.nz/gpacts/public/text/1999/an/142.html* The codes of recommendations and minimum standards for the welfare of animals, endorsed as a national code on 25 May 1995 *http://www.maf.govt.nz/mvg/scientif/scientif.htm#E143E1*
Netherlands Minister of Health, Welfare and Sport	Dutch experiments on animals act entered into force on 5 February 1997 *http://prex.las.vet.uu.nl/nca/*
Norway Ministry of Agriculture	Norwegian Animal Welfare Act, 1974 *http://oslovet.veths.no/dyrevernloven.html*

Table Q.1. *Continued*

Country, Institutions	Legislation
Republic of Czechia Czech National Assembly	"Law for the Protection of Animals" no 246 Sb, 15. April 1992, inclusive the amendments no. 162 Sb, 19. May 1993
South Korea 1. Ministry of Agriculture, Fishery and Forestry 2. Ministry of Health and Welfare	1. "Animal Protection Act", May 1991 2. "Pharmaceutical Affairs Law" Korean Good Laboratory Practice Guidelines for Breeding and Caretaking of Test Animals
Spain	Real Decreto 223/1988 *http://www.boe.es*
Sweden The Swedish National Board for Laboratory Animals (CFN) Ministry of Agriculture, Stockholm	National Board for Laboratory Animals Ordinance Amending the Board's Ordinance (LSFS 1988:45, as last amended by SFS 1998:1344 of October 22, 1998) Containing Regulations and General Recommendations Concerning Ethical Examinations of the Use of Laboratory Animals for Scientific Purposes", etc. *http://www.algonet.se/~stifud/act-ordinance.html*
Switzerland	"Tierschutzgesetz vom 9. März 1978 (TSchG) "Tierschutzverordnung", 27. Mai 1981 *http://www.bvet.admin.ch/*
Taiwan Council of Agriculture; In future: Provincial/Municipal Governments	no legislation, regulations or policies in place; animal protection law drafted
United Kingdom	"Protection of Animals Act", 1991 "Animals (Scientific Procedures) Act", 1986 *http://www.homeoffice.gov.uk/ccpd/aps.htm*
U.S.A. U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS), Animal Care (AC)	Guide for the Care and Use of Laboratory Animals, January 1996 *http://www.nap.edu/readingroom/books/labrats/* The Animal Welfare Act, signed into law in 1966, amended four times (1970, 1976, 1985, 1990), can be found in United States Code, Title 7, Sections 2131 to 2156 *http://www.nal.usda.gov/awic/legislat/usdaleg1.htm*
Most countries	Local ethical commisions at universities and other institutions of biomedical research

Q.2 Techniques of blood collection in laboratory animals[3]

Q.2.1 Introduction

Blood is collected from laboratory animals for various scientific purposes, for example, to study the effects of a test drug on various constituents, such as hormones, substrates, or blood cells. In the field of pharmacokinetics and drug metabolism, blood samples are necessary for analytical determination of the drug and its metabolites. Blood is also needed for some *in vitro* assays using blood cells or defined plasma protein fractions.

The techniques for blood collection depend on specific factors which differ from one experiment to the other. There is a difference between terminal and non-terminal blood collection techniques. The conditions of blood collection at the end of an experiment which includes death of the animal (terminal experiment) are completely different (anesthesia, volume of blood) from those of single or repeated blood collections from a conscious animal. Terminal blood collection under anesthesia allows the use of techniques which are not acceptable for non-terminal blood collections.

Q.2.2 Aspects of animal welfare

Minimizing any pain and distress in laboratory animals during the procedure have to be as important as achieving the desired experimental results. This is important not only for humanitarian reasons but also as part of good scientific practice. Blood collection may be stressful to the animal due to the handling and the discomfort associated with a particular technique. Many biochemical and physiological changes are associated with stress which affect the results, e.g. increases in the blood levels of catecholamines, prolactin and glucocorticosteroids can influence certain metabolic parameters, such as glucose, as well as the counts of erythrocytes, white cells, and packed cell volume. Therefore, stress should be reduced to an absolute minimum if it is not possible to avoid it at all, this is not only in the interest of animal welfare but also in the interest of good science to obtain representative data. To minimize stress during blood collection, e.g.

[3] By A. W. Herling, J. Maas, K. Seeger (1st edition), revised by U. Albus (2nd edition).

from dogs or cats, it may be helpful for the animal as well as for the operator first to do some dummy runs and provide rewards to the animal.

During non-terminal blood collection it is important not to withdraw too much blood which could reduce total blood volume and lead to false results. A reduced total blood volume is accompanied by a reduced hemoglobin content and oxygen transport capacity (Gainer et al. 1955) as well as by a fall in blood pressure, and an increase in the concentrations of stress-related hormones. It may be further accompanied by other factors such as necrosis of the gastric mucosa.

The welfare of the individual animal should not be endangered by removal of too large a volume of blood or by too frequent collections. This may be the case more often when small laboratory animals, e.g. mice, gerbils, rats or hamsters are used. In these cases the study protocol should be adapted to use more animals to minimize distress for the individual animal.

Q.2.3 Total blood volume

The total blood volume is very difficult to determine (McGuill et al. 1989) and depends on species, sex, age and health as well as nutritional condition. Total blood volume is smaller in larger animals than in smaller animals of the same species in relation to body weight. It is also smaller in older and obese animals compared to normal weight and young animals. Total circulating blood volume is in the range of 55–70 ml/kg body weight.

Q.2.4 Terminal blood collection

Terminal blood collection represents (i) exsanguination as a single process of blood removal to collect as much blood as possible and (ii) multiple blood sampling during a terminal experiment under general anesthesia. Basically, exsanguination should only be performed after the animal has been rendered unconsciuos by another method, e.g. physical stunning or general anesthesia. This is due to the fact that stress occurs with extreme hypovolemia and accessing deeper blood vessels causes pain. Due to the anesthetized condition of the animal and the terminal nature of the experiment, methods can be used for exsanguination which can never be recommended for non-terminal blood collections with recovery of the animal. These include

- blood withdrawal from the V. cava caudalis or the aorta after laparatomy when as much blood as possible should be removed in a sterile manner,
- exsanguination after decapitation, incision of the jugular vein or carotid artery or techniques in the slaughterhouse, when a non-sterile collection is possible,
- retro-orbital bleeding of smaller laboratory animals like mice, gerbils, hamsters and rats which can also be a method of exsanguination.

Q.2.5 Non-terminal blood collection

Non-terminal blood collections can be differentiated into single and multiple blood withdrawals. Possible peripheral veins for blood withdrawal are listed in Table Q.2.

Q.2.5.1 Single blood removal

A single withdrawal of up to 15% of total blood volume does not influence the well-being of the animal. However, the removal of 15 to 20% might be accompanied by side effects such as fall in cardiac output or blood pressure. Haemorrhagic shock can be induced by the withdrawal of 30–40% of total blood volume and the loss of 40% causes mortality in up to 50% of pigs and rats (McGuill et al. 1989).

A single removal of up to 15% of total blood volume may be repeated after 3–4 weeks from normal and healthy animals with no detectable adverse effects. This does not mean that the animal does not experience any adverse effects, but it does not show any.

Symptoms of hypovolaemic shock are fast pulse, pale mucous membranes, hyperventilation and a subnormal body temperature including cold skin and extremities. In these animals therapeutic intervention consists of volume substitution with warm isotonic intravenous infusion.

Q.2.5.2 Multiple blood removal

Multiple withdrawal of blood samples should not exceed 1% of total blood volume every 24 h (0.6 ml/kg/d). More frequent withdrawals and/or removal of larger volumes of blood causes anemia.

Symptoms of anemia are pale mucous membranes of the conjunctiva or inside the mouth, intolerance to exercise and an increased respiratory rate in cases of severe anemia. Anemia can be easily detected by determination of erythrocyte cell count and packed cell volume (haematocrit), hemoglobin level as well as reticulocyte count in a blood sample. In case of anemia

the animal should be treated with iron and vitamin B12 and should be monitored for the above mentioned blood parameters during therapy until normal values are reached again.

Q.2.6 Technical aspects of blood removal

A common method in mice and rats for collecting up to 0.1 ml capillary blood is to remove the tip of the tail. For repeated blood sampling the blood clot on the tail has to be removed to get fresh capillary blood. This method is sufficient for multiple blood collections to determine, e.g. blood glucose or total radioactivity after the administration of radiolabeled drugs. In tail-less animals such as guinea pigs and hamsters, cardiac puncture under general anesthesia may be the preferred technique.

Blood collections from larger animals will preferably be performed from a superficial vein. The person holding the animal and raising the vein plays a key role in collecting blood without undue stress to the animal by talking to and stroking the animal. Some animals, e.g. dogs and some primates, may be trained to present a limb for blood removal without the use of any physical restraint.

It is important to locate the vessel accurately before insertion of the needle or the catheter. In most cases obstruction of the venous return is necessary for distension of the vessel and to successfully insert the needle. The bore of the needle should be as large as possible to ensure rapid blood withdrawal with minimal risk of blood clotting within the needle. When the sample is taken too quickly by a syringe, the vein will collapse. After the needle has been withdrawn, continuous pressure should be applied immediately to the puncture site and maintained for at least 30 s. The animal should be monitored 15 min later to check for after-bleeding.

Q.2.6.1 Permanent venous cannulation

For multiple blood collections a permanent venous access by chronic cannulation is often recommended. In most cases, particularly in rats, it is necessary to restrain the animal in harnesses or jackets to prevent it from damaging or removing the cannula. In these cases the signs of stress are often apparent by an increase in serum levels of stress hormones. However, a few days after implantation of catheters, hormone levels are normal in restrained rats (Tsukamoto et al. 1984; Wiersma and Kastelijn 1985). Such animals are usually housed alone and the tethering restricts normal movements such as lying on the back and rolling over. Such restrictions may be considered as potential sources of stress. This can be prevented by having the catheter exit the back of the animal for only 2 cm and capping it with a steel needle. At the time of the experiment, a longer catheter is attached for blood collections.

A simple device for serial blood collection has been described by Sir-Petermann et al. (1995).

One has to balance very carefully the distress and discomfort of the individual animal with a permanent cannulation under restraint conditions for a longer period with multiple blood samplings without permanent cannulation. In the first case, multiple values from the same animal can be obtained showing perhaps individual differences among a group of animals. In the latter case it may be necessary to use a larger number of animals but there is less discomfort for the individual animal.

Short-term cannulation (less than a day) of a peripheral blood vessel in larger animals is easy to perform. A butterfly needle can be inserted under aseptic conditions and multiple blood samples can readily be collected. Long-term cannulation (longer than two days) in larger and smaller animals often presents complications such as blockage of the cannula by thrombi. The infusion and administration of substances via the permanent cannula are much easier than the removal of blood. Thrombi attached to the end of the cannula function as a one-way valve. Clotting can be prevented by repeatedly filling the catheter with saline containing heparin.

Q.2.6.2 Retro-orbital bleeding

Blood sampling by orbital puncture is a controversial technique. The puncture of the orbital venous plexus is often performed in tail-less animals, e.g. hamsters. This technique is also used in rats and mice, when larger volumes are required which cannot be obtained from the tail vein. Basically, retro-orbital bleeding should always be performed under anesthesia. Pasteur pipettes, micropipettes or microcapillary tubes are used and pushed with a rotating movement through the conjunctiva laterally, dorsally or medially of the eye to the back wall of the orbit. In general, inflammatory reactions can be seen histologically in the puncture track four days after punction. After 4 weeks the lesions have healed without detectable scars (van Herck et al. 1992). However severe side-effects such as retro-orbital haematoma with subsequent pressure on the eye cannot be completely excluded. This pressure can dam-

age the optical nerve. The animal may be unable to close its eye. Bleeding from the orbital venous plexus should only be performed with recovery of the animal in exceptional circumstances when there is no other method available. The technique should be performed only by a well-trained staff and only one eye should be used.

Q.2.6.3 Cardiac puncture

The collection of blood by cardiac puncture has been performed in guinea pigs, gerbils and hamsters. In these species it is difficult to collect blood by alternative methods except retro-orbital bleeding. In general, cardiac puncture should be performed under general anesthesia with atropine as premedication to prevent cardiac arrhythmia. If cardiac puncture is used for a non-terminal blood withdrawal with recovery, the animal has to be separated from other animals until it is fully conscious. It should be carefully watched for adverse effects and sacrificed if found in distress due to complications like bleeding into the pericardium or into the thorax.

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Table Q.2. Blood vessels for venous blood withdrawal

Species	V. coccygica + tt	V. auricularis	Orbital venous	V. jugularis	V. cephalica V. saphena	V. femoralis	V. mammarica
Mouse	cc + cc	–	a	–	–	–	–
Gerbil	cc + cc	–	aa	a	–	–	–
Hamster	–	–	aa	a	–	–	–
Rat	ccc + cc	–	a	aa	–	–	–
Guinea pig	–	c	–	a	–	–	–
Rabbit	–	ccc	–	c	–	–	–
Cat	–	–	–	cc	ccc	a	–
Dog	–	–	–	ccc	ccc	a	–
Rhesus monkey	–	–	–	aa/c	ccc	cc	–
Pig	–	iii	–	iii/cc(cvc)	–	–	–
Sheep	–	–	–	ccc	c	c	–
Goat	–	–	–	ccc	c	–	c
Cattle	cc	c	–	ccc	c	–	cc
Horse	–	–	–	ccc	–	–	–

c/cc/ccc	conscious animal	c/i/a	possible alternative
i/ii/iii	immobilized animal	–	not recommended or impossible
a/aa/aaa	anaesthetized animal	cvc	Cranial vena cava
ccc/iii/aaa	recommended route	tt	Amputation tail tip
cc/ii/aa	acceptable route	Repetition of letters indicates the preferred condition	

Q.3 Anesthesia of experimental animals[4]

Q.3.1 Introduction

In biomedical research, experiments should only be done with a conscious animal if it is not possible to do the study in an anesthetized one. Anesthetic conditions should always be chosen to exclude stress, discomfort and pain for the animal which could have negative influences on the pharmacological results and reproducibility of the data. Therefore, an experimental design causing minimal discomfort to the animal is always preferable. This is important not only for humanitarian reasons but also for good scientific practice.

Many pharmacological experiments are performed under anesthesia:

- terminal experiments under anesthesia followed by euthanasia,
- experiments under anesthesia with recovery at the end of the study, and
- experiments in which an animal is surgically prepared under anesthesia and continuation of the experiment occurs with the conscious animal after recovery.

Generally, two possibilities exist for immobilization of aggressive animals and to prevent escape: (i) physical restraint (e.g. immobilization cages or immobilization tubes) and (ii) chemical restraint with anesthetic compounds. As a rule of thumb it is recommended to use physical restraint for animal studies in which no anesthesia would be used in comparable studies in man. Physical restraint can be used for short and painless interventions like administration of substances or blood sampling from a vein.

In general, physical restraint produces fear, distress and anxiety in experimental animals with the result of stress symptoms which could affect the results of the study. To minimize pain for the animals, to obtain correct and reproducible results and to protect the handlers from aggression by the animals, it is often necessary to use chemical restraint. However, the chemical used can affect the biochemistry or physiology of the animal.

It is possible to anesthetize special areas of the animal (local anesthesia) or the whole animal (general anesthesia). Local anesthesia plays only a minor role for experimental animals as compared to general anesthesia.

[4] By J. Maas, A. W. Herling, K. Seeger (first edition), revised by U. Albus (second edition).

Q.3.2 Local anesthesia

Local anesthesia is the regional and reversible elimination of pain with chemical compounds. Circulatory, pulmonary and renal functions are not disturbed and the animals are conscious. Surface anesthesia has to be distinguished from the anesthesia produced after a local injection. The most common compounds for surface anesthesia are tetracaine and proparacaine. Procaine, butanilicaine, lidocaine, mepivacaine and etidocaine are commonly used injectable local anesthetics. Local anesthesia is only recommended for gentle and calm animals (cattle, sheep). For most laboratory animals, general anesthesia is the method of choice.

Q.3.3 General anesthesia

Q.3.3.1 Preparation

It is very important to check the general condition of the animal prior to anesthesia. This check should include a clinical examination (inspection, auscultation, palpation) of the animal concerned. Sometimes it could be useful to perform a few laboratory tests, e.g. hematocrit, hemoglobin, pH-value of blood and acid/base parameters.

Those animals with a vomiting reflex should be fasted prior to anesthesia. Most animal species should be fasted for a period of at least 12 h but pigs and cattle for at least 24 h. Water can be offered during the fasting period ad libitum.

Q.3.3.2 Premedication

Premedication is recommended prior to anesthesia for easier administration of the anesthetic and for elimination of side effects of the anesthetic used, such as disturbing autonomic reflexes.

Q.3.3.2.1 Hydration and base excess

Based on hematocrit, hemoglobin and erythrocyte values, the hydration of the animal should be normalized prior to anesthesia. Infusions of glucose or Ringer solution can be used for this purpose. To check the suc-

cess of the treatment repeated determinations of the above mentioned values are necessary.

In cases of acidosis (pH of blood <7.36), treatment of the animals with $NaHCO_3$ is recommended. If measurement of base excess is possible, the amount of $NaHCO_3$ can be determined from the following formula:

$$\text{Dose } NaHCO_3 \text{ (ml)} = \text{g body weight} \times \text{base excess} / 0.6$$

Q.3.3.2.2 Atropine

To avoid cardiopulmonary problems and to decrease saliva production, atropine should be administered intramuscularly prior to general anesthesia. The recommended dose varies considerably and is usually between 0.05 and 0.1 mg/kg body weight. Cats and rodents have a higher activity of atropine-esterase in the liver, and these species need higher amounts of atropine (up to 0.25 mg/kg).

Q.3.3.2.3 Sedation and pain elimination

Indications for sedation and elimination of pain are to calm the animals and to stabilize the autonomic nervous system.

For sedation the following compounds are used:

- minor tranquilizers without autonomic effects:
- meprobamate, diazepam,
- major tranquilizers with autonomic side effects:
- propionyl-promazine, acetylpromazine, azaperone, dehydrobenzperidol, xylazine, detamidone.

Anaesthesia does not necessarily lead to analgesia (elimination of pain). Although general anaesthesia produces loss of consciousness and pain is not perceived, the noxious stimuli will be transmitted to the CNS and will develop central hypersensitivity, leading to a postoperative heightened perception. To reduce the degree of central hypersensitivity analgesics have to be administered before noxious stimulation begins (pre-emptive analgesia). Analgesia should also reduce or eliminate peripheral inflammation, which aggravates central hypersensitivity.

For analgesia, the opioids used are mainly:

- methadone, meperidine, fentanyl.

In most species (dog, rabbit, guinea pig) a sedative effect is to be observed after administration of these compounds. In other species (pig, cat) an excitatory effect can occur.

Minor tranquilizers, major tranquilizers and analgesic compounds are often used in common with anesthetics. A compilation of such combinations is summarized in Table Q.3.

Q.3.3.3 Course of anesthesia

The animal has always to be observed very carefully during anesthesia. Various systems can be checked with technical equipment, e.g. circulatory system (heart rate, pulse, blood pressure, ECG, peripheral perfusion, temperature) or pulmonary system (respiratory rate).

A very important procedure during anesthesia is the determination of the depth of anesthesia. There are four stages of anesthesia:

I. Stage of analgesia (from the first effect to unconsciousness):
   - heart and respiratory rate increase, normal dilation of pupils.

II. Stage of excitation (from the beginning of unconsciousness to the start of regular respiration): respiration irregular, dilated pupils, increased motor reflexes, nystagmus, opisthotonus.

III. Stage of tolerance (from the beginning of regular respiration to the termination of spontaneous respiration):
   This stage is divided into four steps:
   A) regular respiration, narrow pupils, most reflexes present
   B) skeletal muscles relaxed, narrow pupils, no eyelid reflex, corneal reflex present, flat respiration, good analgesia.
      This is the optimal stage of anesthesia for surgery
   C) only corneal reflex present, respiration very flat, pupils dilated
   D) no reflexes, respiration very flat, pupils very dilated

IV. Stage of asphyxia (after termination of the spontaneous diaphragmatic respiration):

V. no reflexes, no respiration: danger of death, immediate use of antidotes is necessary to prevent death.

These various stages appear clearly in the case of ether. By using combinations of different anesthetics – mainly by using combinations with muscle relaxing agents – the reactions of animals will differ from this scheme.

Q.3.3.4 Routes of general anesthesia

In general, there are two different routes to induce general anesthesia: (i) injection and (ii) inhalation anesthesia. Sometimes combinations of both routes are used. The decision for one or the other route depends on the animal species, the purpose of the study and the necessity of control during anesthesia.

Q.3.3.4.1 Injection

By using this route of anesthesia the narcotic compound is dissolved in a liquid. The route of administration can be intravenous, intramuscular, subcutaneous or intraperitoneal. The mostly frequently used compounds are mentioned below:

Barbiturates

There are three groups of barbiturates: long acting, short acting and very short acting barbiturates. For laboratory animals short and very short acting barbiturates are used predominantly (sodium pentobarbitone, thiopental, hexobarbital).

Barbiturates are metabolized in the liver and mainly excreted via the bile. They are very fat soluble. Their short duration of action is caused by a distribution into adipose tissue. Fat represents a large compartment for these compounds with a relatively slow excretion. This can lead to prolongation of the narcotic effects after repeated dosing. The dosing of barbiturates should be adjusted according to the observed reactions of the individual animal as there are individual differences due to age, body weight, size, fat content and general condition of the animal. Barbiturates are not analgesic and should not be given without opioids.

Chloralhydrate

Chloralhydrate is a relatively old soporific compound. By using it for anesthesia cardiovascular side effects are often observed. The range of dosing is very narrow. Its use for laboratory animals is therefore limited. Intraperitoneal injections in rats can lead to paralysis of the ileus.

Combinations of analgesic with neuroleptic compounds

This method is often used for dogs and rodents. Strong analgesics (morphine, methadone, meperidine, fentanyl) are combined with neuroleptics like phenothiazine, acetylpromazine or butyrophenone. The anaesthesia can be rapidly terminated by available antagonists.

Ketamine

Ketamine is a neuroleptic compound with a very fast onset of action following intramuscular administration. It can be used for nearly all species. A side effect of this compound is an increased tonus of skeletal muscles but this can be prevented by the simultaneous administration of xylazine or diazepam.

Hypnotic agents

Hypnotics are compounds which produce a very deep sleep without analgesia (metomidate). Therefore combination with neuroleptic compounds is recommended (e.g. combination azaperone with metomidate for pigs). As a single compound, metomidate can only cause anesthesia in birds.

Xylazine

Xylazine is frequently used for anesthesia in combination with other substances (Table Q.3). As a single compound it is only used to produce anesthesia in cattle.

Urethane

Urethane was formerly used as a hypnotic agent, It can, at the appropriate dose, produce a long acting (about 10 h) anesthesia in rats. Urethane is liver toxic and therefore its use is limited to some pharmacological models in which liver metabolism is of no importance. Due to its carcinogenic properties it should be avoided wherever possible.

The important criteria of anesthesia are sedation, unconsciousness, analgesia and relaxation. These cannot be achieved with a single compound. Therefore, a combination of different compounds is necessary. The most common combinations for different species are listed in Table Q.3 with respect to the duration of anesthesia: short (up to 30 min), medium (up to 120 min) and long anesthesia (longer than 120 min).

Q.3.3.4.2 Inhalation

This kind of anesthesia plays only a minor role for small laboratory animals like rodents. Inhalation anesthesia is more common for the bigger laboratory animals such as dogs, cats, sheep, goats and monkeys. The advantages of this form of anesthesia are the possibilities of controlling exactly the depth of anesthesia and of fast management of complications.

The parts of an inhalation system include:

- Bottle with oxygen (blue bottles)
- Valve to regulate pressure (reduces the pressure of the oxygen-bottle)
- Flowmeter (monitors the gas flow to the animal)
- Evaporator (evaporation of liquid anesthetic compounds)
- Oxygen-bypass (fast supply of oxygen to the animal in case of need)
- Tube to the system

Different techniques are used for laboratory animals:

Technique of insufflation
Administration of anesthetic compounds is performed via a mask. Expiration occurs into the air of the room. Advantages are the simple procedure without valves and CO_2-absorber, and the very small dead volume of the system. Disadvantages are the waste of compounds, drying of the trachea of the animals, the impossibility of checking the respiration volume and the expiration of narcotic compounds into the room air (jeopardy to the staff).

Open system
Inspired and expired gases are separated by a valve. The inspired air consists of the fresh mixture of gases. The expiration reaches completely the atmosphere. The "Stephen slater" is the most used system of this group. It is recommended for smaller animals.

Half-closed and closed systems
In closed systems all of the expired air passes to a CO_2 absorber. The CO_2 is removed chemically and the air is inspired again with newly evaporated anesthetic compounds mixed with oxygen. In a half-closed system, part of the expired air reaches the atmosphere. Advantages of closed systems include the economic benefit, the decrease of fluid and body heat loss from the animal and no risk to the laboratory staff. Disadvantages are the necessity to change the absorber every 8–10 h during anesthesia, the production of heat and the increase of resistance to breathing.

Summary
If it is possible inhalation should be done by intubating the animal. The risk of aspiration of stomach contents with the danger of an aspiration pneumonia can then be minimized. It is very important to use a tube with the correct diameter and length. An animal should be unconscious for intubation (see Table Q.3). In order to avoid gulp or cough reflexes it is recommended to administer succinylcholine, a muscle relaxant. Atropine can also be administered to decrease saliva production. Generally, all methods of injection anesthesia mentioned (Table Q.3) can be combined with an inhalation method. Such a "balanced anesthesia" is recommended for long and highly sophisticated operations.

Q.3.3.4.3 Inhalation compounds

The inhalation mixture has to include 21% oxygen. Sometimes it is better to administer 33% oxygen. Despite the danger of explosion ether is one of the most frequently used anesthetic compounds, similarly, isoflurane, methoxyflurane and enflurane are widely used compounds for inhalation anesthesia. By using a mixture of N_2O and O_2 the amount of the evaporated compounds can be reduced drastically (Table Q.4).

Q.3.3.5 Termination of anesthesia

Inhalation anesthesia can be stopped by removing the supply of evaporated compounds. To hasten the elimination of anesthetic compounds, the concentration of oxygen in the system can be increased for a period of five min.

The elimination of injected compounds is difficult to influence. It may be possible to accelerate metabolism of the anesthetic by using agents which stimulate metabolism in the liver and excretion by the kidney.

It is very important to check the body temperature of the animal during and after anesthesia. In cases of low body temperature the use of heating lamps or pads is necessary. After termination of anesthesia the animals go through the same phases as mentioned above but in the reverse order (tolerance, excitation, analgesia).

During anesthesia it might be necessary to stimulate respiration or circulation. Stimulatory agents for respiration are doxapram, pentamethylentetrazole, nikethamide, methetarimide, lobeline or micoren. Stimulatory agents for circulation are adrenaline, effortil, dopamine and ephedrine. The application of pure oxygen via a mask is also recommended during an injection anesthesia. Antidotes to morphine and its derivatives are morphine-antagonists like naloxone. Yohimbine is an antagonist of xylazine. The antidote for diazepam is flumazenil. There are no direct antagonists for ketamine and barbiturates.

Table Q.3. Anesthesia of experimental animals (values are in mg/kg)

Species	Premedication	Sedation	Short anaesthesia	Medium anaesthesia	Long anaesthesia
Rat	Atropine (0.2 s.c.)	Diazepam (2.5 i.m.)	Alfentanyle + Etomidate (0.03 + 2 i.m.) or Inhalation (Isoflurane)	Xylazine + Ketamine (5 + 100 i.m.) or Pentobarbitone (50 i.p.)	Xylazine + Ketamine (16 + 100 i.m.) or Urethane (1 500 i.m.)
Mouse	Atropine (0.1–0.25 s.c.)	Diazepam (5 i.p.)	Alfentanyle + Etomidate (0.03 + 2 i.m.) or Inhalation (Isoflurane)	Xylazine + Ketamine (5 + 100 i.m.) or Pentobarbitone (50 i.p.)	Xylazine + Ketamine (16 + 100 i.m.)
Hamster	Atropine (0.1–0.2 s.c.)	Diazepam (5 i.p.)	Inhalation (Isoflurane or Ether)	Xylazine + Ketamine (5 + 50 i.m.) or Pentobarbitone (35 i.p.)	Xylazine + Ketamine (10 + 200 i.m.)
Guinea pig	Atropine (0.1–0.2 s.c.)	Diazepam (2.5–5 i.m.)	Inhalation (Isoflurane)	Xylazine + Ketamine (2 + 80 i.m.)	Xylazine + Ketamine (4 + 100 i.m.) or Pentobarbitone + Chloralhydrate (30 i.p. + 300 i.v.)
Rabbit	Atropine (0.1–0.2 s.c.)	Diazepam (1–5 i.m.)	Inhalation (Isoflurane)	Xylazine + Ketamine (5 + 25–80 i.m.)	Xylazine + Ketamine (5 + 100 i.m.) or Pentobarbitone + Chloralhydrate (30 i.p. + 300 i.v.)
Cat	Atropine (0.05–0.2 s.c.)	Diazepam (0.2–1 i.m.)	Acetylpromazine (0.5–1 i.v./i.m.) or Propionylpromazine 0.5–1 i.v.) or Xylazine (2 i.m.)	Xylazine + Ketamine (2 + 10 i.m.) or Ketamine (5 i.v.) or Inhalation (Isoflurane)	Pentobarbitone (35 i.v./i.p.)
Dog	Atropine (0.05 s.c.)	Xylazine (3 i.m.) or Acetylpromazine (0.5 i.m.) or Propionyl-promazine (0.5 i.m.) or Droperidol (1 i.m.) or Diazepam (1 i.m.)	Thiopental (17 i.v.) or Metomidate + Fentanyl (4 + 0.005 i.m.) or Alfentanil + Etomidat (0.03 + 1 i.m.) or Inhalation/Intubation (Isoflurane)	Xylazine + Methadone (2 + 1 i.m.) or Xylazine+Ketamine (2 + 10 i.m.) or comb. with Diazepam (0.6 i.m.) or Propionylpromazine + Methadone (0.5 + 1 i.v.) or Acetylpromazine + Methadone (0.5 + 0.5–1 i.v.)	Pentobarbitone (30 i.v.) or Xylazine + Ketamine (2 + 15 i.m.) or Intubation (Isoflurane)
Pig		Azaperone (1–2 i.m.) or Chlorpromazine (1–2 i.m.)	Thiopental (10 i.v; 5 % solution)	Azaperone + Metomidate (0.05–5 + 2.5–5 i.m. + i.v./i.p.) or Tiletamine + Zolazepam + Xylazine (2 + 2 + 0.5–1 i.m.)	Pentobarbitone (10–25 i.v.)
Sheep Goat		Xylazine (0.05–0.1 i.m.) or Diazepam (2 i.m.)	Xylazine + Ketamine (1–2 + 5–10 i.m.) or Thiopental (7.5–10 i.v.)	Pentobarbitone (20–30 i.m.)	Ketamine after pretreatment with Xylazine/ ketamine 3–4 h or Intubation (Isoflurane)
Monkey[a]	Atropine (0.05–0.1 s.c.)	Diazepam (1.0 i.m.) or Xylazine (1–2 i.m.) or Ketamine (10–30 i.m.)	Inhalation/Intubation (Isoflurane)	Xylazine + Ketamine (2 + 10 i.m.)	Pentobarbitone (20–30 i.v./i.p.) or Intubation (Isoflurane)

[a] Anaesthesia of monkeys depends very much on the monkey species.

Table Q.4. Inhalation compounds and their characteristics

Compound	Conc. with N_2O/O_2 (%)	Conc. without N_2O/O_2 (%)	MAC
Methoxyflurane	0.4–1	3.5	0.23
Isoflurane	1.0–2.5	1.5–3.5	1.4
Enflurane	0.5–1.5	2	2.2
Ether	3.5	5–10	3.2

MAC: minimal alveolar concentration for an anesthetic effect.

Q.3.4 Postoperative analgesia

To effectively reduce pain in animals a pain assessment has to be made using behavior, stress response etc. Pain assessment will be facilitated by

- a good knowledge of the species specific behaviors of the animal being assessed
- a knowledge and comparison of the individual animal's behaviour before and after the onset of pain (eg pre- and post-operatively)
- the use of palpation or manipulation of the affected area and assessment of the responses obtained
- examination of the level of function of the affected area: e.g. leg use following injury or limb surgery, together with a knowledge of any mechanical interference with function
- the use of analgesic regimens or dose rates that have been shown to be effective in controlled clinical studies, and evaluation of the change in behaviour this brings about
- a knowledge of the non-specific effects of any analgesic, anaesthetic or other drugs that have been administered

Analgesics can be broadly divided into two groups, the opioids or narcotic analgesics and the non-steroidal anti-inflammatory drugs (NSAID). Clinical pain involves several pathways, mechanisms and transmitter systems. To provide the most effective pain relief, drugs of different classes should be applied, acting on different parts of the pain system, for example combining opioids and NSAIDs.

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Q.4 Euthanasia of experimental animals[5]

Q.4.1 Introduction

Biomedical research needs animals. This is most obvious in case of *in vivo* animal experiments. However, for other scientific purposes, e.g. *in vitro* studies, biological material is also necessary to study enzymes, membranes, receptors, cells, tissues, or organs which are obtained from dead animals. Therefore, animals have to be sacrificed in biomedical laboratories (i) at the end of an *in vivo* experiment, (ii) during experiments where sacrifice of the animals is not part of the study but must be done when pain, distress and suffering exceed acceptable levels or if it is likely for the animal to remain in pain or distress after cessation of the experiment, and (iii) to provide biological material for *in vitro* studies.

The following remarks are a summary of the *Recommendations for Euthanasia of Experimental Animals* of the *Commission of the European Communities* (1993) and the *Guidelines for Skillful and Human Euthanasia of Laboratory Animals* (1993) of Switzerland.

Q.4.2 Euthanasia

Euthanasia means a gentle death and should be regarded as an act of a human method of sacrificing an animal with a minimum of physical and mental suffering. The method of euthanasia should be appropriate for the species and the age of the animals. The method should be painless, avoid excitement and achieve rapid unconsciousness and death. Additionally, the method should be reliable, reproducible and irreversible.

Prior to euthanasia, it is important to recognize symptoms of fear, distress and anxiety; these symptoms are species specific. Depending on the species these symptoms may include distress vocalisation, attempts to escape, aggression, freezing, salivation, urination and defecation. Distress vocalization and release of certain odors or pheromones by a frightened

[5] By A. W. Herling (1st edition), revised by U. Albus (2nd edition).

animal may cause anxiety in other animals housed nearby. In this context it has to be stressed that many vocalisations of animals are in a range of frequencies which are out of the human hearing range. Therefore, animals should not be present during euthanasia of other animals, especially of their own species. If possible, an animal should not be killed in a room where other animals are housed, in particular in case of a bloody method of euthanasia, e.g. decapitation.

Euthanasia usually requires some physical control over the animals. Suitable control minimizes pain, distress, fear and anxiety in the animal and depends on animal species, size, state of domestication and method of euthanasia. Gentle handling, stroking and talking to the animal during euthanasia often have a calming effect on many animals. The use of sedating and immobilising drugs may be necessary in those cases, where capture or restraint may cause pain, injury or anxiety to the animal.

The person performing euthanasia is the most relevant factor during sacrificing an animal in order to cause a minimum of pain, fear and distress. A suitable method of euthanasia can be extremely harmful to the animal if it is badly performed. All persons performing euthanasia should be well trained, demonstrate professionalism and be sensitive to the value of animal life.

After euthanasia it is essential to confirm death. Signs of death are cessation of heartbeat and respiration, and absence of reflexes. Death must be guaranteed by exsanguination or removal of the heart, destruction of the brain, decapitation, evisceration or the presence of rigor mortis.

Methods for euthanasia of laboratory animals can be separated into physical and chemical methods.

Q.4.2.1 Physical methods recommended for euthanasia of laboratory animals

Physical methods are stunning (concussion, electrical stunning, and stunning with a captive bolt), cervical dislocation, decapitation, and microwave irradiation. The different methods of stunning as well as cervical dislocation cause a rapid loss of consciousness which must be followed immediately by a method to force and guarantee death of the animal.

Concussion may be sufficient in smaller animals, e.g. rodents, to achieve unconsciousness and is performed by a blow to the head. Electrical stunning is a common method in the slaughterhouse predominantly for pigs. Only specific equipment must be used for this method either in the slaughterhouse or in laboratory. Stunning with a captive bolt is also a common and an effective method for larger animals in the slaughterhouse to achieve unconsciousness. Adapted equipment can also be used for larger rabbits in biomedical laboratories. The correct localisation of the captive bolt is important in order to achieve immediate destruction of the brain. Cervical dislocation destroys the brainstem but the large vessels to the brain are often intact. All these methods have to be followed immediately by an act to force and guarantee death, e.g. exsanguination, removal of the heart or destruction of the brain.

During the decapitation process the head is separated from the neck which causes an immediate interruption of the blood circulation to the brain and a fall in blood pressure in the brain with subsequent loss of consciousness. This is valid only for warm-blooded animals. In cold-blooded vertebrates it is recommended to stun the animals prior to decapitation due to their higher resistance against anoxia. For decapitation of smaller laboratory animals specific guillotines have been developed.

Euthanasia by microwave irradiation is used by neurobiologists for fixation of brain metabolites without destruction of brain anatomy. Only specific equipment developed for this purpose must be used (no domestic microwave ovens). It is essential to localize correctly the microwave beam onto the brain of the animal.

Q.4.2.2 Chemical agents recommended for euthanasia of laboratory animals

Many chemicals can cause death due to their toxicity, but only a few are recommended for euthanasia. The most suitable chemicals for euthanasia are certain anesthetics in overdose. In this case, the anaesthetic agent causes unconsciousness, followed by death.

Volatile anesthetics such as halothane, enflurane, isoflurane and methoxyflurane should only be used in a gas scavenging apparatus. Carbon dioxide at high concentrations of 80 to 100% causes unconsciousness within a few seconds.

Injectable anesthetics, predominantly barbiturates such as sodium pentobarbitone, are the most widely used and the most appropriate agents for euthanasia for most animals. Three times the anesthetic dose causes generally rapid unconsciousness and death. Intravenous injection is the most reliable and rapid route. Intraperitoneal injection may also be used in smaller rodents but it needs more time for death to occur. The intracardial and intrapulmonal administration can only be recommended in unconscious animals, because it is painful and, in the case of intracardial injection, difficult to perform successfully on the first attempt.

The agent T61 is a mixture of a local anesthetic, a hypnotic and a curariform component. It is used only intravenously. Due to the curareform component it is not allowed in some countries but it has been demonstrated that unconsciousness and neuromuscular blockade occur simultaneously in dogs and rabbits. Nevertheless, prior sedation should be performed if possible.

Q.4.2.3 Methods and agents not to be used for euthanasia of laboratory animals

Physical methods not to be used for euthanasia are exsanguination, rapid freezing, pithing, decompression, hyperthermia, hypothermia, asphyxia, drowning and strangulation. Chemicals not to be used are carbon monoxide, nitrogen, nitrous oxide, cyclopropane, chloroform, trichloethylene, hydrogen cyanide, magnesium sulfate, potassium chloride, nicotine, strychnine, chloral hydrate, and ethanol. Some of the above mentioned chemicals are not recommended for euthanasia because they are extremely noxious and dangerous to the experimenter.

Neuromuscular blocking agents such as curare, succinylcholine or suxamethonium which do not cause rapid unconsciousness prior to death should also not be used. Ketamine is a very good anesthetic with a wide therapeutic safety margin for most animal species. Therefore, it is unsuitable for euthanasia.

However, non-acceptable methods of euthanasia can be used if animals are anaesthetized or rendered insensible and unconscious by a recommended method. This is used for, e.g. exsanguination, rapid freezing, and pithing. Exsanguination must not be performed in sight or smell of other animals. Rapid freezing is important to minimize enzymatic processes prior to subsequent biochemical determinations in tissues and organs. Pithing is a quick method of brain destruction achieved by insertion of a needle through the foramen magnum.

Q.4.2.4 Recommended methods for euthanasia for specific animal species

Mouse
- Decapitation
- Cervical dislocation with subsequent exsanguination
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 150 mg/kg i.p.

Rat
- Concussion, cervical dislocation (both with subsequent exsanguination), and decapitation, conducted only by well trained persons
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 100 mg/kg i.v. or 150 mg/kg i.p.
- Microwave irradiation

Hamster
- Decapitation; conducted only by well trained persons
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 300 mg/kg i.p.

Guinea pig
- Concussion (with subsequent exsanguination), and decapitation; conducted only by well trained persons
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 150 mg/kg i.p.

Rabbit
- Stunning with captive bolt
- Concussion (with subsequent exsanguination); conducted only by well trained persons
- Sodium pentobarbitone at a dose of 120 mg/kg i.v.
- T61 at a dose of 0.3 ml/kg strictly i.v. via a catheter

Cat
- Sodium pentobarbitone at a dose of 100 mg/kg i.v. or 200 mg/kg i.p.
- T61 at a dose of 0.3 ml/kg strictly i.v. via a catheter; it is recommended to anesthetize the animal beforehand with 20–30 mg/kg ketamine i.m. or 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.

Dog
- Sodium pentobarbitone at a dose of 100 mg/kg i.v.
- T61 at a dose of 0.3 ml/kg strictly i.v., it is recommended to anesthetize the animal beforehand with 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.

Ferret
- Sodium pentobarbitone at a dose of 120 mg/kg i.p.

Cattle, sheep, goat, horse, pig
- Sodium pentobarbitone at a dose of 100 mg/kg i.v.; for larger animals prior sedation is recommended (Xylazin).

- All other acceptable methods which are used for slaughtering

Primate

- Sodium pentobarbitone at a dose of 100 mg/kg i.v.; it is recommended to anaesthetize the animal beforehand with 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.
- T61 at a dose of 0.3 ml/kg strictly i.v., it is recommended to anesthetize the animal beforehand with xylazine/ketamine i.m.

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