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BetaSys

Systems Biology of Regulated
Exocytosis in Pancreatic β -Cells



Systems Biology

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Preface

Aside from the obvious statement that it should be a theory capable of unifying all our knowledge about insulin secretion, in both health and disease, not much is known about a *systems biology* of regulated exocytosis in pancreatic β -cells.

Let us recall common knowledge: Patients with diabetes suffer from an absolute or relative lack of the hormone insulin. Insulin is produced by pancreatic β -cells and secreted by *regulated exocytosis*. In type 1 diabetes (juvenile diabetes) β -cells are destroyed by autoimmune mechanisms. In type 2 diabetes, and pre-diabetic states, we observe a decline in β -cell function.

There has been a great deal of experimental work over the last 50 years, and a fair amount of mathematical modelling since the 1980s, but the systems biology approach is new and not fully developed. Genome-wide scans for diabetes genes have pointed to promising candidates involved in β -cell function, raising the importance of systems issues to a new level.

This book gives a snapshot of the field at the threshold of a possible explosion in knowledge. We introduce recent advances in observational techniques, ranging from genetic epidemiology via proteomics to multi-parameter cell sensing, MRI, ET and nanoparticle-based cell imaging. We summarize what these techniques have revealed regarding β -cell function: the generation of huge new data sets, dealing with ions, DNA, proteins, electrical phenomena, cell membranes, cell organelles and tissue, in extreme spatial and temporal scales from Ångström to micrometres and from picoseconds to minutes and hours.

Because it is an exciting area of research, there are many new ideas about the systems biology of insulin secretion, but they often diverge to such an incredible degree that it seems impossible to decide which of the many possible directions one should pursue.

The division of the text into five overlapping parts reflects the duality between the medical pull and the technological push originating from model-based measurements and mathematical modelling, estimation, control and simulation: The *clinical and pharmaceutical need* of a systems biology approach is to go beyond umbrella diagnosis and solely symptomatic non-individualized treatment – by distinguishing different levels and different traits of functioning within a comprehensive picture of the disease(s). The technological push towards systems biology is based on the

design and use of the so-called *mathematical microscope*. By that term we denote general and/or specific mathematical methods to:

1. interconnect local and global, small and asymptotic phenomena;
2. specify bio-medical ideas and processes;
3. expand heuristically the imagination by theorems and simulations;
4. guide new experiments and exploit traditional experiments more efficiently, and,
5. identify mechanisms and parameters.

In Part I, the medical scene is presented. Systems biology of β -cells is introduced; established facts and open questions of the focused systems analysis are summarized; tutorial reviews on mitochondria and metabolic signals, on β -cell ontogenesis and on the role of the cytoskeleton in transport and release of insulin-containing granules are given. We close this part describing the ideal (up to now mostly a hope) of the aforementioned mathematical microscope, i.e. the replacement of lengthy, expensive, and ethically worrying, in vivo experiments by in vitro-tuned computer simulations.

In Part II, we give five tutorial reviews on new developments in imaging and sensors, emphasizing magnetic resonance imaging, electron tomography, in vivo applications of inorganic nanoparticles, sensor-based assays and bioimpedance spectroscopy.

In Part III, four tutorial reviews are devoted to DNA variations, genetically programmed defects, proteomic analysis and the role of islet amyloid polypeptide in the pathogenesis of type 2 diabetes.

In Part IV, physiological, pharmaceutical and clinical applications are addressed by three tutorial reviews: one on the present state of islet transplantation; one on predictive protein networks and the identifications of drug targets; and one on nanotoxicity.

In Part V, different examples of well-established and developing applications of mathematical modelling and numerical simulation in β -cell analysis are demonstrated. We begin this part with a discussion of the silicon cell paradigm of making experiment-based computer replicas of parts of a biological system, and a presentation of a novel class of mesoscopic simulations probing cellular dynamics. We show how rigorous harmonic analysis raises doubt about metabolic oscillations, and present two mathematical models of minimal complexity able to assess β -cell function in an individual. In the closing chapter on geometric and electromagnetic aspects, we wish to show the heuristic use of mathematical modelling and the recourse to first principles, namely to generate radically new hypotheses for future verification – or falsification.

Beyond our interest in presenting systems biology approaches to understanding and curing diabetes mellitus, our specific “story” is intended to provide a worked case of a systematic teaching of the basics of systems biology, namely how to overcome the three basic challenges, met wherever systems biology is demanded:

1. Interconnect the multiple levels: diabetes syndromes, β -cell function, membrane processes, intracellular dynamics, proteomics and genome mapping.

2. Bridge multiple scales: DNA, plasma membrane, insulin granules, cells and islets.
3. Learn to collaborate in a multi-disciplinary environment.

The chapters of this book were written as tutorial reviews for a broad audience of students of human biology, informatics, mathematical biology and medicine. The level was chosen for teaching graduate classes, studying in Ph.D. programmes and postdoctoral training. For two chapters, namely [Chapters 8 and 20](#), additional material is provided on the Internet for the convenience of students, and in order to compensate at least to some extent for the 2D display limit of print media when used to illustrate 3D image information. The major caveat is, of course, that the extremely fast progress of the field makes one run the risk of presenting already obsolete viewpoints at the time of use of the textbook. Therefore, when we describe the state of the art, we emphasize the principles involved. In such a way the book shall serve as a companion also for work going forward.

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Part I
Systems Biology Approach to β -Cells

Chapter 1

Systems Biology of the β -Cell – Revisited

Flemming Pociot

Nature is fond of hiding herself
Heracleitus

Abstract The insulin-secreting β -cell is one of the most specialized cell types. Almost the entire intracellular machinery is directed towards maintaining glucose homeostasis. It has been a focus of intensive research for several decades, which has culminated in the characterization of processes involved in synthesis and secretion of the hormone in considerable details. The stage of knowledge of this cell is reflected in a substantial variety of mathematical models and numerical simulations that aim to explain major aspects of the β -cell function (see other chapters). These models, though answering many questions about the β -cell function, remain to be only isolated attempts and have not yet been integrated into a single more unified model. Thus, there is a need to apply a holistic approach.

Keywords β -cell · Diabetes · Genetics · Networks · Systems Biology

1.1 Introduction

Cells are complex biological systems that consist of components that interact with each other, under regulatory strategies, in response to internal and environmental signals.

A *biological system* can be viewed as a set of diverse and multi-functional components (genes, gene products, and metabolites), which population level changes over time in response to internal interactions and external signals. The interactions among the system components reflect the value one component has on values of other components. These interactions are usually governed by a set of biophysical

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laws, most of which are only partially known. Modelling involves inference of both the interaction map (*structural inference*) of the system and the mathematical formalisms that approximate the dynamic biophysical laws the system follows (*dynamic inference*) [1]. Both of these approaches aim at characterizing the system at different levels of abstraction and neither of them are trivial.

System biology requires exact knowledge of magnitudes of kinetics parameters that characterize the components involved. This knowledge has so far been incomplete, thus limiting the use of all models suggested. Further, development of such models into the direction of systems biology requires that the model in effort be closely tight to innovative and exact experimentation.

Understanding how cellular components interact in time and space is crucial for deciphering the functions inside a living cell. Technological advances make simultaneous detection of thousands of biological variables possible. Microarrays are used to measure expression of thousands of genes simultaneously, yeast two-hybrid (Y2H) and affinity purification-mass spectrometry (AP-MS) assays are used to map protein interactions, and chromatin immunoprecipitation (ChIP)-chip methods are used to identify interaction between proteins and DNA, just to name a few.

The challenge will be to integrate such existing data with data such as the role of ion channels in creating the electric activity in the β -cell membrane, the traffic infusion of insulin granules with plasma membrane, and the role of glycometabolism and mitochondria, to obtain precise data from living cells and to include the dynamic nature of these processes (see other chapters) (Fig. 1.1). That will allow us to formulate a comprehensive and robust model of the main networks of biochemical and physical processes involved in insulin secretion. Such a model is expected to be a valuable tool in understanding β -cell function and the development of disease – i.e. diabetes – related to β -cell dysfunction. It may also open avenues to finding novel ways of treatment modalities. Additionally, the models may be of use for testing effects of various pharmacological agents.

1.2 The β -cell and Diabetes

Diabetes is common and getting more common and is now one of the most common non-communicable diseases globally (see also Box “The History of Diabetes”).

Diabetes is a life-threatening condition. More than 250 million people live with diabetes and the disease is associated with enormous health costs for virtually every society. It is estimated that 3.8 million men and women died from diabetes in 2007, more than 6% of the total world mortality. It is further estimated that the number of people with diabetes will reach 380 million in 2025 [2]. This means that 1 out of 14 adults worldwide will have diabetes in the year 2025. It is estimated that the world spent at least USD 232 billion in 2007 to treat and prevent diabetes and its complications [3, 4]. Diabetes is certain to be one of the most challenging health problems in the 21st century.

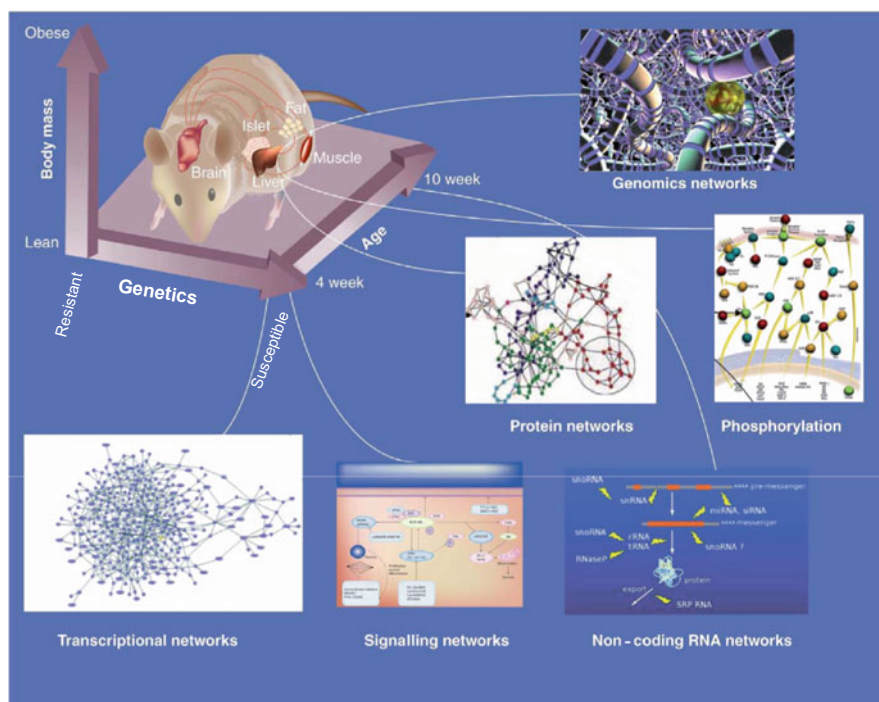


Fig. 1.1 Large-scale molecular, clinical, and imaging data provide the ability to capture the complexity of interacting molecular networks both within and between tissues that underlie complex phenotypes. Reproduced from *Pharmacogenomics* (2009) 10(2):203–212, with permission from Future Medicine Ltd.

Diabetes mellitus is classified on the basis of etiology and clinical presentation of the disorder into four types: (1) type 1 diabetes, (2) type 2 diabetes, (3) gestational diabetes mellitus, and (4) other specific types [5, 6].

In type 1 diabetes the β -cells of the pancreas are destroyed by the immune system for reasons not fully understood and little or no insulin is produced. The disease can affect people of any age, but usually occurs in children and young adults.

Type 2 diabetes is characterized by insulin resistance and relative insulin deficiency. The specific reasons for developing these abnormalities are not known in details. In type 2 diabetes, β -cell deterioration occurs due to a combination of genetics, low-grade inflammation, and glucose- and lipo-toxicity [7, 8]. The diagnosis of type 2 diabetes usually occurs after the age of 40 years, but could occur earlier especially in populations with high diabetes prevalence.

Gestational diabetes mellitus is a carbohydrate intolerance of varying degrees of severity, which starts or is first recognized during pregnancy. Women who have had gestational diabetes mellitus have increased risk of developing type 2 diabetes in later years [9].

Other specific types of diabetes includes monogenic forms with most of these affecting β -cell functions (i.e. maturity-onset diabetes of the young (MODY)) [10] (see Chapter 13).

The insulin-producing tissue is known as the islets of Langerhans. There are approximately one million islets in a normal human pancreas. They are named after the German pathologist Paul Langerhans (1847–1888) who discovered them in 1869 [11]. There are five types of cells in an islet where the most abundant (60–80%) cell type is the β -cell that produces insulin [12]. The glucose metabolism is under strict control. Despite intake of large amounts of carbohydrates or several days of starvation, plasma glucose levels are maintained within a very narrow window. Insulin is a key regulator of the glucose homeostasis (Fig. 1.2).

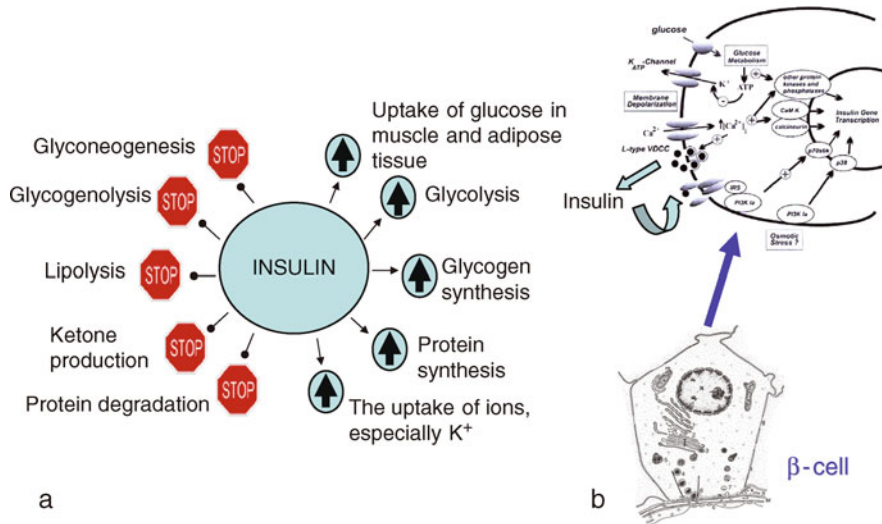


Fig. 1.2 (a) Insulin has several metabolic and cellular effects. (b) Glucose-induced insulin production and secretion is a tightly controlled process, which is schematically outlined only in the figure.

The cell can be considered an open system exchanging material with its environment. In this sense, a living entity has a dynamic relationship with its surroundings, and to fully understand β -cell function, it might be critical to study simultaneously the other cell types of the islet of Langerhans. This has not been addressed thoroughly, i.e. few studies have evaluated β -cell function as part of a biological system comprising all islet-cell types, despite the fact that islets are often used for experimental studies as opposed to isolated β -cells. The cell provides spatial organization through its membranes and other structures and much of this is not encoded in DNA. It could be argued that various interactions between molecules are defined by the laws of chemistry, and that if one can determine which biomolecules are supplied by the information given in DNA, then one can deduce the behaviour of the cell.

1.3 Genetics of Diabetes – From GWA to NWA Studies

Both type 1 and type 2 diabetes are polygenic, multi-factorial diseases, i.e. several genes contribute to disease risk, which in combination with environmental factors may cause clinical disease, whereas MODY forms are monogenic.

Recently, very large genetic studies, so-called genome-wide association (GWA) studies, have revealed a large number of susceptibility genes in both type 1 and type 2 diabetes [13, 14]. Interestingly, several of the potential candidate genes might be implicated in β -cell function. In MODY forms, all known disease genes are directly involved in β -cell function [10]. Chapters 12 and 13 deal with this in detail. Here it suffices to say that the fundamental aim of genetics is to understand how an organism's phenotype is determined by genotype and implicit in this is predicting how changes in DNA sequence are affecting the transcriptome, the proteome and the metabolome (Fig. 1.3).

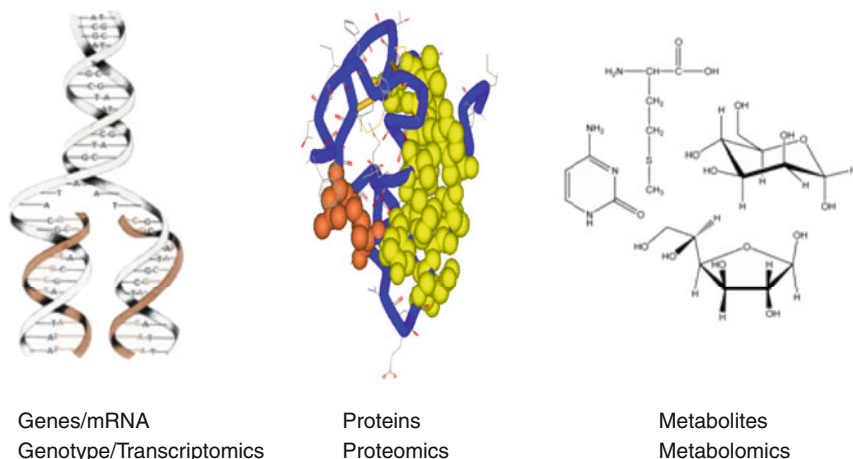


Fig. 1.3 Changes in DNA sequence may lead to alterations in the transcription process e.g. by affecting splice sites or introducing stop codons. Other variations will cause amino acid substitutions resulting in structural changes or altered physical properties of the protein. Other disparities in DNA sequence may cause chemical modifications of the protein leading to changes in biological function.

GWA studies encompass a number of challenges, which include (1) statistical power; (2) biological interpretation, e.g. which gene is the “right” one; and (3) the fact that many genes may interact to confer disease risk [15]. The latter has not been thoroughly addressed in current GWA scans. Nevertheless, GWA studies provide a rapid and high coverage method to map genetic interaction networks at large scale, although this is often not recognized. For detailed discussion of GWA studies data, see Chapter 12.

Additionally, a simple linear interpretation of DNA information may no longer be sufficient. For example much of the genome is transcribed producing many functional non-coding transcripts [16, 17]; and higher-level structures and processes in

the cell, such as nuclear organization, the structure of DNA, and chromatin remodelling, are intrinsic to transcription regulations [18]. So although DNA is vital and central to heritable information, this information has limited meaning except in the context of the cell and the additional rules and codes that it provides.

A new approach to classify human disease that both appreciate the uses and limits of reductionism and incorporate the tenets of the non-reductionist approach of complex system analyses is therefore essential. Obviously, all disease phenotypes reflect consequences of variation in complex genetic networks operating within a dynamic environmental framework. Cellular networks are modular, consisting of groups of highly interconnected proteins responsible for specific cellular functions. Disease represents the perturbation or breakdown of a specific functional module caused by variation in one or more of the components producing recognizable developmental and/or physiological dynamic instability. Such a model offers a simple hypothesis for the emergence of complex or polygenic disorders: A phenotype often correlates with inability of a particular functional module to carry out its basic function. For extended modules, many different expression combinations of perturbed genes might incapacitate the module, as a result of which variations in expression of different genes may lead to the same clinical phenotype. This correlation between disease and functional modules, i.e. moving from GWA to NWA (network-wide (pathway) association) studies, can also help in understanding cellular networks by identifying which genes are involved in the same cellular function or network module [19, 20]. Importantly, this association of disease with functional modules may also influence our choice of rational therapeutic targets. It may also tell us which perturbations are deleterious and which are not.

The History of Diabetes

1550 BC	Egyptian physician Hesy-Ra of the Third Dynasty makes the first known mention of diabetes – found on the Ebers Papyrus – and lists remedies to combat the “passing of too much urine”.
600 BC	Indian physician Sushruta identified diabetes and classified it as <i>Medhumeha</i> , “honey-like urine” in Sanskrit. He further identified it with obesity and sedentary lifestyle, advising exercises to help cure it.
250 BC	The name “Diabetes” is attributable to Demetrius of Apamea and is derived from the Greek word <i>diabeinein</i> , to go to excess.
120 AD	Greek physician Aretaeus of Cappadocia gives the first exhaustive medical description of symptoms using the name “diabetes”.
1010	Central Asian scholar Avicenna provided a detailed account on diabetes mellitus in <i>The Canon of Medicine</i> , “describing the abnormal appetite and the collapse of sexual functions”, and he documented the sweet taste of diabetic urine. Like Aretaeus before him, Avicenna recognized primary and secondary diabetes.
1425	Diabetes first appears in the English language in a medical text as the Middle English word “diabete”.

16th Century	Swiss physician Philipus Aureolus Paracelsus – considered the “Martin Luther of Medicine” – identifies diabetes as a serious general disorder.
1674	In his treatise <i>Pharmaceutice rationalis</i> , Thomas Willis added the word “ <i>mellitus</i> ”, from Latin meaning “honey” as a reference to the sweet flavour of urine in diabetes.
1871	French physician Apollinaire Bouchardat notices the disappearance of glycosuria in his diabetes patients during food rationing of food under the Siege of Paris in the Franco-Prussian War, and formulates individualized diets to treat the condition.
1889	Oskar Minkowski and Joseph von Mering demonstrate how removing a dog’s pancreas produces diabetes.
1919–1922	The discovery of insulin, see Box “The History of Insulin” in Chapter 17 .
1923	Insulin is made commercially available.
1936	Harold Himsworth states that diabetes falls into two types based on “insulin insensitivity”. This discovery later leads to the diabetes classifications of type 1 and type 2.
1967	First successful pancreas transplantation performed at the University of Minnesota.
1993	After 10 years of clinical study, the Diabetes Control and Complications Trial (DCCT) report is published and clearly demonstrates that intensive therapy delays the onset and progression of long-term complications in individuals with type 1 diabetes.
1998	The United Kingdom Prospective Diabetes Study (UKPDS) scientifically links the control of glucose levels and blood pressure control to the delay and possible prevention of type 2 diabetes.
1999	First successful islet transplantation program at the University of Alberta, Canada. The procedure becomes known as The Edmonton Protocol.
2006	The United Nations recognizes diabetes as a global threat and designates World Diabetes Day, 14 November – in honour of Frederick Banting’s birthday – as a UN Day to be observed every year starting in 2007.

Further Reading:

Gale EA (2001) The discovery of type 1 diabetes. *Diabetes* 50:217–226

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Added by the editors

1.4 Why Systems Biology?

A systems biology approach aims to devise models based on the comprehensive qualitative and quantitative analyses of diverse constituents of a cell or tissue, with

the ultimate goal of explaining biological phenomena through the interaction of all its cellular and molecular components (see also Box “Ludwig von Bertalanffy (1901–1972)”).

This is based on the analysis of large-scale datasets, such as those generated by DNA microarrays and proteomics. The model is subsequently refined through introduction of perturbations in the system and a new round of large-scale gene/protein analysis. System biology is thus an interactive process in which researchers propose models based on large datasets, make predictions departing from the model, and then conduct additional large-scale experiments to test the prediction and refine the model.

As system biology progresses, multi-factorial diseases, such as diabetes, may be understood in terms of failure of molecular components to cooperate properly. Consequently, multi-factorial diseases may be approached and treated in a much more rational and effective way [21]. The starting point for this is the notion that any biological property is the result of the interaction in time and space of a large set of different molecules, cells, organs and/or organisms. The iterative cycle of model-driven experimentation with experimental data-driven modelling, in combination with novel systems analysis tools, constitutes the very heart of system biology. Biological systems are endowed with two features of great interest: *function* as an emergent property and *robustness* [22]. A function derives as an emergent property when it is not present in the individual components of the systems, but emerges when the various parts interact following an appropriate organizational design. Robustness is the ability to maintain stable functioning despite internal and external perturbation. Robustness is not absolute and cells are, in general, robust in the face of frequently occurring perturbations but fragile when dealing with rare events. Moreover, robustness has a cost in terms of allocation of resources, e.g. to glucose sensing, insulin synthesis and secretion. The evolutive acquisition of robustness appears to be one main source of complexity for biological systems.

1.5 Systems Biology – How?

To identify the structure and function of intracellular networks of the β -cell, it is important to keep in mind that the β -cell is a well-organized system having its own components strategically positioned and regulated in a functionally independent modular manner.

This form of internal organization has been selected throughout evolution and further by differentiation to successfully carry out the increasing complexity of maintaining glucose homeostasis – but also as a “safety switch” where diverse reactions can take place without being deleterious to the cell. Connectivity among such functional modules is the key feature that makes the cell operate as an integrated system, allowing internal functions to influence one another. Identifying functional modules is thus crucial for understanding intracellular functions.

Ludwig von Bertalanffy (1901–1972)

This Austrian-Canadian philosopher and theoretical biologist has been the originator of the *general systems theory*. Essential for Bertalanffy's system concept is the possibility of connecting various systems, then called *subsystems*, into a larger system. The *wiring* schedule for these connections constitutes a network. Pairs of categories can be specified, like thing–environment, part–whole, simple–complex, structure–function, cause–effect, and process–development.

Attempts to formulate a mathematized *General Systems Theory* were not successful. On the contrary, special systems theories considering separate aspects have emerged and proved worthwhile, in particular the mathematical theories of control, regulation, and optimization, of dynamical systems and stochastic processes, and of automata, signal and information.

Approaching biological objects as organized dynamic systems, Bertalanffy advocated an *organismic* conception based on the vague idea of *holistic integrity*. Today, a half century after Bertalanffy, it seems that he has underestimated the value and the necessity of accumulating single facts – and partly exaggerated the unifying role of “First Principles” as we know them from physics, partly subdued to the socio-biologist spirit of his time and origin.

For molecular and cell biology, a lasting achievement is Bertalanffy's theory of *open systems*. He considered the most simple biological systems which yield all three life performances: homeostasis, reproduction, and information gathering. He emphasized that such systems must stand in continuing energetic, material, and information exchange with their environment. He concluded that they are thermodynamically open systems which – typically – approach a state of *flow equilibrium*. While closed systems approach the equilibrium state asymptotically, there can and will appear *false states* and *overflows* in open systems.

Further Reading:

Schimming R (2003) Back to Bertalanffy: the system theoretical approach to biology. EMTB – Eur Commun Math Theor Biol 5:11–15, July 2003

Added by the editors

In systems biology, basically two main approaches are considered for this, the *bottom-up* and the *top-down* approaches [23, 24]. The bottom-up approach (from modules to networks) is basically a reductionist method and strongly promoted by the concepts and technology of biochemistry and molecular biology. The concept of this approach is the idea of initially aggregating detailed biological knowledge about individual components and quantitative information about the molecular interaction into appropriate molecules and then to interconnect these into architectures suitable for holistic analysis of the system of interest. Depending on any frame work of choice, e.g. deterministic or stochastic, continuum or discrete modelling approaches, the first step involves verbal level modelling, where necessary information about the system is collected. This is followed by the model setup and subsequent solution of equations, performing parameter sensitivity analysis. This process yields sufficient information about new experimental designs, which can then be used for

the quantification of individual components and their dynamic behaviour. Parameter estimation can then be followed which paves the way for the testing and validation of the model. The final result is cycled until a satisfactory result is obtained. This modelling cycle is the key to the success of bottom-up or reductionist model building.

One example of the bottom-up approach to systems biology is the Silicon Cell Programme (see [Chapter 19](#) and <http://www.siliconcell.nl>) [25]. In the Silicon Cell Programme metabolic pathways like glycolysis models are built from kinetics rate laws in vitro. In vitro measurements of enzyme kinetics allow for an exact characterization and manipulation of quantitative parameters and will yield a reasonably steady-state depiction of glycolysis. Although, the reductionist approach is powerful in building logically simple hypothesis and devising ways to test them, it is very difficult to reconstitute a model for a whole biological system by combining the pieces of information it generates. Using a reductionist approach, the entire system model must be reconstituted by combining information about every molecular step in the system. Any missing pieces of information may block the reconstitution of the system. Therefore, the bottom-up approach requires essentially complete information including the dynamic behaviour of each step, to build a system model. Also, reductionism by definition focuses on information essential to a simplify question and intentionally discards extra information. The major difficulty in applying this strategy, however, is the definition of criteria for the demarcation of these modules to guarantee a certain level of autonomy. For the time being these modules are most often defined from an empirical, textbook-driven decomposition of the network into subsystems performing particular physiological functions. Because of the absence of a rigorous definition of these subunits, the question remains whether the fundamental organization of the biological networks or multi-organ systems is modular at all or distributed, or whether it is probably best described as being a little bit of both.

The top-down approach is basically linked to a high throughput reductionisms (e.g. assigning biological function to the genome of an organism). Another aspect, however, is characterized by exhaustive, simultaneous descriptions of biological systems such as global profiling (transcriptome, proteome, metabolome, interactome, fluxome, etc.). Such broad and detailed information about a biological system provides us with a view different from reductionism – a view of how the system behaves as a whole. In a top-down approach, the primary focus is planning an execution of large-scale experiments to generate a lot of information about the genome, proteome, metabolome, etc. The experimental design is therefore a crucial part that determines whether this strategy will be successful or not. Perturbation experiments are performed and followed by the design of further experiments, new time theories, etc. Next step involves the large-scale data generation of “omics” data and following data analysis new networks are inferred, which give an idea about the structure and interaction between the players in the system and a general impression of its performance.

There is also a possibility of studying the modularity in such reconstructed networks by studying the interaction of sub-networks within the networks and pinning down their autonomous nature or lack of it. The approach is useful but only if

its pitfalls are appreciated. One example is the use of Bayesian networks (which assumes the absence of feedback) for those biological regulatory networks that are known to abound in feedback. A second example is the common description of cellular regulation only in terms of gene networks, although it is clear that proteins, signal transduction and metabolism are involved in this regulation in addition to mRNA. An example of a top-down approach is the study of the gut microbial-mammalian interactions on the metabolic profiles of the host organism [26]. Here, the application of metabolomics has revealed specific metabolic phenotypes associated with different microflora [24]. This illustrates that an important source of metabolomic variability in the host will be missed if only the host genome is studied.

However, there should be no controversy about the need of a mixed complementary approach, but only about the relative importance in context with the existing knowledge related to a given problem. The two models pursue different goals: A bottom-up model is constructed to be locally correct (describing individual reactions by correct rate laws and parameters), while a top-down model, on the other hand, is optimized for a good global fit to *in vivo* behaviour. In a model of limited size, it is unlikely that both requirements will be fulfilled at the same time. Once the problem has been formulated, the purpose and the scope of the model and the related known information about the different aspects of structure, and regulation of the system can be studied. If the known outweighs the unknown, then the bottom-up approach can be taken with confidence. But in the case where there are a large number of unknowns the top-down approach is the logical way to bridge the gap between the knowns and the unknowns. The ultimate goal of such a hybrid approach is that the characterization of the behaviour of parts of the system should be consistent with the expected and/or observed behaviour of the system as a whole. The top-down approach is to deconstruct the system into smaller parts. The bottom-up approach is to reconstitute elemental steps into larger parts. If the result of these approaches meets in the middle, and if they are consistent in terms of links between modules, multiple functions of elements, etc., we can be confident that we are on the right track. In other words, we can use information from the reductionist approach as constraints in large-scale model building and vice versa. This endeavour is possible only with strong coordination between experimental and modelling efforts. It is important that both areas are tightly linked and function in tandem as one single effort.

Biological networks that have been studied extensively usually consist of many intermediate steps between the initial response (to a signal) and the outcome. We do not know all these steps and components for any complex system, but a simplifying assumption can be made by recognizing that different parts of the network operate at a different speech. For example, kinases operate on a much faster time scale than gene regulation. Then when interpreting gene regulation data, one can assume in many experimental settings that the cell signalling network has already responded and is in a steady state, and that one therefore is assaying the events secondary to gene expression. Also there might be events taking place at the same time scale that are not being measured, e.g. chromatin modification during a transcriptomic experiments.

1.6 Challenges of Methodological Advances

A further problem is that the control samples and even the experiments that a scientist may conduct are specific to the lab where they are performed. This creates a challenge for reusing knowledge of how systems behave from experiment to experiment.

Examples of data standardizations relevant to systems biology include Gene Ontology (GO) for describing gene function [27], Minimal Information About Microarray Experiments (MIAME), Systems Biology Markup Language (SBML) [28], and Cell Markup Language (CellML) for describing biomolecular simulations [29], and Minimum Information for Biological and Biomedical Investigations (MIBBI) [28, 30].

A prerequisite to system biology is the integration of heterogeneous experimental data, which are stored in numerous life sciences databases. The most important tool for reaching and understanding of biology at the level of systems is the analysis of biological models. The basic building blocks for these models are existing experimental data, which are stored in literally thousands of databases. It might be a common misconception that the main problems of database integration are related to the technology that is used for these purposes, but it has been argued that although the mastering of such technology can be challenging, the main problems are actually related to the databases themselves [31]. These problems include technical issues as web access, problems with data extraction and lack of software interfaces, problems with data pre-processing, inappropriate conceptualizations, and problems with the content of databases. In addition, social issues and political obstacles may be responsible for some problems with life sciences databases [31].

The dynamics of the system can be mathematically modelled, allowing prediction of the response of the system to genetic and environmental perturbations. Data can be used to construct co-expression networks in which the nodes are transcript levels and the edges represent correlations between transcripts. Such models are based on the assumption that genes with correlated expression are likely to be functionally associated (although other explanation such as linkage and/or linkage disequilibrium or the impact of the clinical treatment could also result in correlations). It is also clear that many functionally associated genes would not be correlated given that much regulation is post-transcriptional. Thus, such networks are clearly approximations of the underlying biology, and integration with other datasets and approaches is important. Nevertheless, groups of genes or modules identified by co-expression modelling are significantly enriched for functionally related genes [32].

1.7 Summary

We believe that considerable effort should now be devoted to examine the regulated exocytosis in pancreatic β -cells by a broad perspective rather than focusing narrowly on individual pathways or components. This will require

the application of interdisciplinary approaches including genetics, genomics, proteomics, metabolomics, physiology, and mathematical modelling. This should eventually enable the development of a holistic picture of the β -cell, integrating information from multiple scales, including genes, a transcript, proteins, organelles, and cell and tissue communications.

1.8 Understanding Pancreatic β -cell Death in Type 1 Diabetes – A Systems Biology Approach

Clinically, type 1 diabetes is diagnosed when 70–80% of β -cells have been lost due to immune-mediated destruction [33]. The slow destruction of β -cells, coupled with the autoimmune nature of the disease, suggests that type 1 diabetes is potentially preventable [34].

Do we understand in details how β -cells are progressively killed by the immune system in type 1 diabetes in order to allow a targeted intervention to prevent β -cell loss? And is current research approaches focusing on individual pathways adequate to inform our understanding of this? Currently, the answer to both questions is unfortunately “no”.

When β -cells are exposed in vitro to cytokines, they present functional changes which are comparable to those observed in pre-diabetic individuals, i.e. a preferential loss of the first-phase insulin release in response to glucose, probably caused by decrease in the docking and fusion of insulin granules to the β -cell membrane [35] and a disproportionate increase in the proinsulin/insulin ratio [36]. Cytokines induce stress-response genes that either protect or contribute to β -cell death. They also down-regulate genes related to β -cell function and regeneration, and trigger the expression of chemokines and cytokines that will contribute to the attraction and activation of immune cells.

In a top-down approach, gene expression studies have identified nearly 700 genes that are up- or down-regulated in purified rat β -cells or insulin-producing INS-1E cells after exposure to cytokines and nearly 2,000 genes modified by cytokines or viral infection in human pancreatic islets [37, 38] (http://t1dbase.org/page/bcgb_enter/display/).

Two transcription factors play key roles for cytokine-induced apoptosis, namely nuclear factor- κ B (NF κ B) (induced by interleukin (IL)-1 β , tumor necrosis factor- α (TNF α)), and STAT1 induced by interferon- γ (IFN γ) [39]. Prevention of NF κ B activation protects β -cells in vitro against cytokines-induced apoptosis, whereas in vivo NF κ B blocking protects β -cells from diabetogenic agents [40]. Intriguingly, NF κ B has mostly anti-apoptotic effect in other cell types [41], and recent observations in non-obese diabetic (NOD) mice indicate that inhibition of NF κ B activation in β -cells accelerates the development of diabetes [42]. Comparison between IL-1 induced NF κ B in β -cells (where the transcription factor has pro-apoptotic effect) and fibroblast (where it has anti-apoptotic effect) shows that cytokine-induced NF κ B

activation in insulin-producing cells is more rapid, intense, and sustained than in fibroblast, leading to a more pronounced activation of the downstream genes [43]. These findings suggest that the NF κ B-mediated anti- or pro-apoptotic effect in vitro is cell and context dependent. Activation of NF κ B in β -cells in vivo will play a pro- or anti-apoptotic role depending on the animal model of diabetes studied and possibly on the time window utilized for the NF κ B inhibition.

Systemic STAT1 depletion protects against diabetogenic agents [44] and spontaneous development of diabetes in NOD mice [45]. This suggests that an imbalance between deleterious and protective mechanisms leads to progressive β -cell loss in type 1 diabetes and that this, to a large extent, takes place inside the β -cells and affects the interaction with the invading cells from the immune system. Thus, it can be speculated that prevention of human type 1 diabetes will require hitting multiple targets, i.e. preventing activation of pro-apoptotic β -cell gene networks, supporting β -cell defence/regeneration and arresting/regulating the autoimmune assaults.

Furthermore, the mathematical language has been applied to describe the dynamics of the early pathogenetic events where interaction between the immune system and the β -cell leads to β -cell dysfunction and development of type 1 diabetes [46]. Still, these attempts are very simple, but seem promising in describing the multi-factorial nature of the disease. A mathematical formalism allows for a more comprehensive description of the biological problem and can reveal non-intuitive properties of the dynamics.

Also animal models of human type 1 diabetes have served a prominent function in the development of current ideas of pathogenesis and approaches to therapy. Despite translational obstacles in going from observations in rodents to human studies, animal models may still be useful in a systems biology approach in order to identify disease-relevant biological pathways and/or interactions between such. The following example serves to illustrate the complexity of spontaneous disease development in one such model, i.e. the BioBreeding (BB) rat, and how simple intervention (perturbation of disease network) may lead to extensive changes in β -cell protein expression pattern. A transplantation model was used since destruction of islets in situ in the pancreas is not synchronized in time and space, and to enable proteomic studies of diabetes development and islet destruction in vivo. Extensive proteomics work has been performed using this model [47]. Although clinical symptoms of (type 1) diabetes are abrupt in both humans and rodents, the clinical presentation is preceded by a period of variable length, during which the islets are inflamed individually and gradually destroyed. In other words, the destruction of islets in situ in the pancreas is not synchronized in time and space. The spontaneous development of diabetes and destruction of islets in situ are mirrored in the transplanted islets, which can be excised for further studies. To provide minimal influence on the spontaneous diabetes development, only 200 neonatal BB diabetes-prone (DP) rat islets were transplanted under the kidney capsule of BB-DP rats (syngeneic transplantation) [47].

Proteome studies demonstrated that β -cell destruction could be characterized by a limited number of highly significant modules of co-expressed proteins (see Fig. 1.4a). Interestingly, these islet protein expression patterns were predictive also

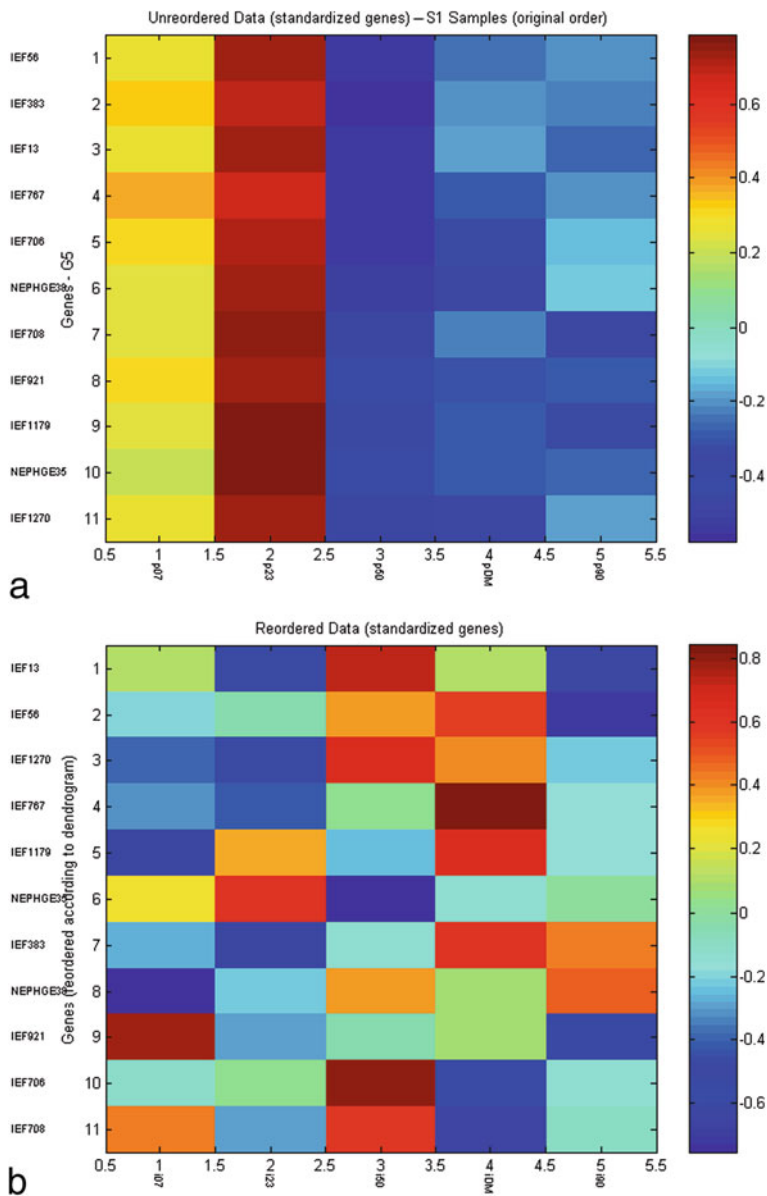


Fig. 1.4 Perturbation of protein expression patterns. Prophylactic insulin prevents or delays diabetes onset and preserves islet transplants in the BioBreeding (BB) transplantation model. See text for details on experimental design. Heat plot of a cluster of protein expressions in transplanted islets excised at different time points post-transplantation (p07: 7 days post-transplantation; p23: 23 days, etc. pDM: at time of diabetes diagnosis). Colour codes are shown to the right of each heat plot. *Red* indicates high expression and *blue* low expression. The Y-axis shows the coordinates of the protein spots identified on a two-dimensional (2D)-gel. Note that the order of proteins is not the same in (a) and (b). (a) Data for spontaneous diabetes development. (b) Data for transplanted BB rats receiving continuous insulin infusion.

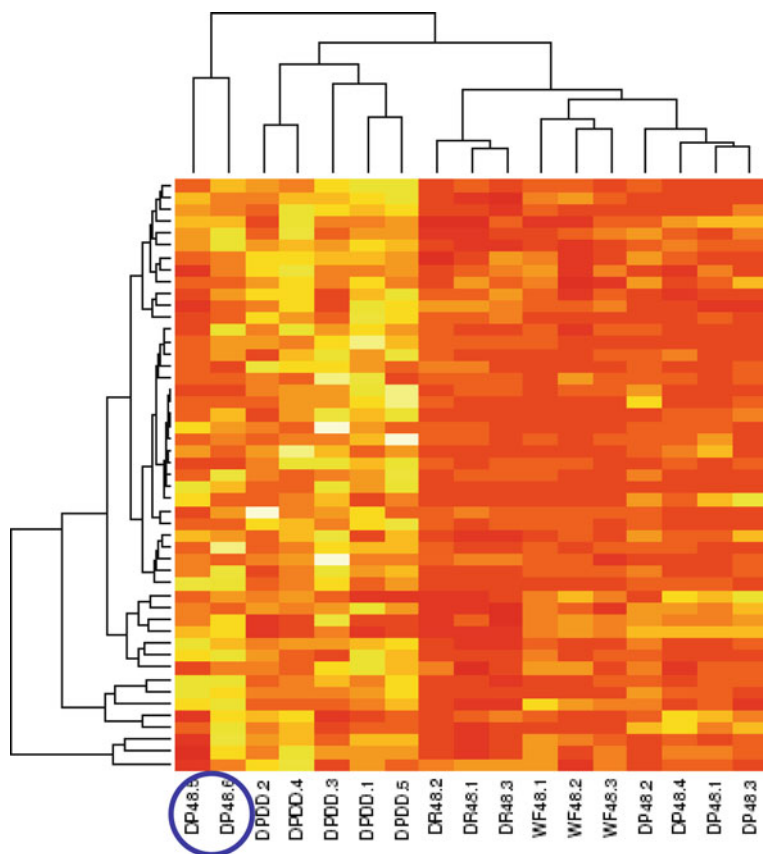


Fig. 1.5 Islet protein expression differs between diabetes-prone (DP) and diabetes-resistant (DR and WF) rat strains and a “diabetogenic” pattern (*left*) and a “non-daibetogenic” pattern (*right*) can be recognized. The hierarchical clustering, on *top*, clearly differentiates between the two groups. *Red* indicates high expression and *yellow* low expression. Each column represents a single animal from which the islet transplant is excised at day 48 after transplantation or at time of diabetes diagnosis, which is around day 48 in this model.

for diabetes development as they could identify and differentiate non-diabetic rats with “diabetic” and “non-diabetic” protein expression patterns (Fig. 1.5).

In a separate study it was concluded that prophylactic insulin treatment administered in this transplantation model considerably decreased the incidence of diabetes and significantly reduced inflammation of the islets *in situ* and in the islet graft [48]. Interestingly, prophylactic insulin treatment led to a substantial perturbation in protein expression patterns (Fig. 1.4b). This illustrates the importance of analysing modules and network interactions of genes and proteins in order to understand and characterize β -cell function (Fig. 1.5).

1.9 Conclusions

Since complex intracellular systems are often composed of smaller, functionally independent sub-network structures, this chapter has discussed different approaches that partition a system into functional modules or reconstruct it based on the interaction between these entities. Different algorithms may result in different compartmentalization of the underlying structure as a whole, but when combined effectively, these approaches should provide a global view of the coordinated functionalities inside complex biological systems as the β -cell.

However, even though a massive amount of experimental data is currently available and substantial biological knowledge has been gained, they remain insufficient for the inference of the missing knowledge, in order to simulate large-scale systems at molecular resolution. There are compromises that, if properly applied, may improve the simulation speed and reduce the dimensionality problem and parameter space, while making only minor sacrifices in the description accuracy of the phenomenon.

The partitioning of the system into functional or mathematical parts is not always a trivial task. Furthermore, when validation or optimization is needed for the sub-models, it should be kept in mind that the data are usually referred to the complete system and not to the parts, which are indeed not independent of the rest of the system. Alternative models, which simulate large-scale systems as a whole by incorporating information and data from genes to proteins and enzymes, are possible when sacrificing dynamic description resolution. Constraint-based models are widely used as top-down models for the investigation of the metabolic capabilities under specific environmental conditions and perturbations, and dynamic phenomena can be approximated by changing the constraints. Additionally, a better way to incorporate other interacting systems such as signal pathways and gene regulatory networks to the complex metabolic network of the β -cell leaves room for improvement towards a multi-level integrated system.

Currently, it is possible to simulate reaction networks occurring in intracellular processes by coupling databases of reaction kinetics to simulation packages for huge systems of non-linear ordinary differential equations (ODEs), e.g. programmes like Silicon Cell, Vertical Cell, E-cell or Cyber Cell. Answering the question of how β -cell dysfunction is related to pathophysiology of diabetes requires an even more geometrical and comprehensive, thoroughly multi-level understanding of living processes based on distributed data over both temporal and spatial scales in combination with systematic extensive experimental measurement of key parameters. The scales range from the single nanometre (nm) to thousands of nanometres and from milliseconds to 5–30 min (see e.g. [Chapters 20–23](#)). Clearly, that requires a distinct type of mathematical modelling and new software for the mesoscale. Although routine in physics, it is not yet available in the biophysical simulation community [49].

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Chapter 2

Established Facts and Open Questions of Regulated Exocytosis in β -Cells – A Background for a Focused Systems Analysis Approach

Erik Renström

Abstract The main task of the pancreatic β -cell is to produce and secrete the blood glucose-lowering hormone insulin. This chapter summarizes current knowledge of the main molecular events involved in that process and follows the chain of events in insulin secretion from synthesis of insulin and its storage in dense core granules and their transport to the cell surface, as well as the molecular reactions that control their fusion with the cell membrane and release of insulin to the blood circulation. These molecular events are discussed on the background of whole-body in vivo insulin secretion pattern, as well as recent advances in the understanding of the pathogenesis of type 2 diabetes. This disease represents one of major health problems associated in economically developing countries, but recently a much improved understanding of the genetic risk for the disease has opened up the prospect of personalized treatment.

Keywords ADRA2A alpha-2A adrenoreceptor · cAMP cyclic adenosine monophosphate · CaV voltage-gated calcium ion channels · DPP 4 dipeptidyl peptidase-4 · Directed granule movement · EPAC2 exchange protein directly activated by cAMP · GLP 1 glucagon-like peptide 1 · Insulin granules · Myosin Va · Kinesins · MSD mean squared displacement · NADPH Nicotinamide adenine dinucleotide phosphate · reduced form · CaV voltage-activated calcium channels · PKA protein kinase A or cyclic AMP-regulated kinase · Random granule movement · RRP readily releasable pool of insulin granules · SNARE soluble N-ethylmaleimide-sensitive factor attachment protein receptor

Abbreviations

(E)GFP	enhanced green fluorescent protein
ADP	adenosine diphosphate
ADRA2A	alpha-2A adrenoreceptor

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ATP	adenosine triphosphate
ATP-ase	enzyme that cleaves and extracts energy from ATP
cAMP	cyclic adenosine monophosphate
CAPS	Ca ²⁺ -dependent activator protein for secretion
Ca _v	voltage-gated calcium ion channels
CIC-3	chloride channel 3
DPP-4	dipeptidyl peptidase-4
EM	electron microscopy
EPAC2	exchange protein directly activated by cAMP
ER	endoplasmic reticulum
GIP	gastric inhibitory peptide, aka glucose-dependent insulinotropic peptide
GLP-1	glucagon-like peptide 1
GLUT	glucose transporter
GTP	guanosine triphosphate
GTP-ase	enzyme that cleaves and extracts energy from GTP
GWAS	Genome-Wide Association Scans
IAPP	insulin amyloid polypeptide
IP ₃	inositoltrisphosphate
K _{ATP} channel	ATP-sensitive potassium ion channel
MODY	Maturity-onset diabetes in the young
MSD	mean squared displacement
munc	mammalian homologue of the unc-18 gene
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
Na _v	voltage-activated sodium channels
NSF	<i>N</i> -ethylmaleimide-sensitive factor
PC	prehormone convertase
PKA	protein kinase A or cyclic AMP-regulated kinase
Rab protein	large family of small GTPases related to the oncogene ras , ras proteins in the brain
Rab27a	rab protein 27a
RRP	readily releasable pool of insulin granules
Slac-2c/MYRIP	Synaptotagmin-like proteins lacking C2 domains/ Myosin-VIIa- and Rab-interacting protein
SNAP-25	synaptosome-associated protein of 25 kDa
SNARE	soluble <i>N</i> -ethylmaleimide-sensitive factor attachment protein receptor
SNP(s)	single-nucleotide polymorphism(s)
TCF7L2	transcription factor 7-like 2; gene with most significant type 2 diabetes SNP to date
TIRF(M)	Total internal reflection or evanescent wave (microscopy)
TNFalpha	tumour necrosis factor alpha
VAMP2	vesicle-associated membrane protein 2 aka synaptobrevin2

2.1 Introduction

Diabetes mellitus is the most common endocrine disorder and incidence rates are increasing worldwide, with an expected doubling in deaths related to diabetes between 2005 and 2030 [124]. The more we learn about this chronic and incapacitating disease, the better we understand how multifaceted it is and that the exact pathogenic mechanisms may differ between individual patients. The disease should be considered an umbrella diagnosis, with the chronic elevation in blood glucose concentrations as the common denominator. One thing that unites all diabetes subtypes is the central role of the failing insulin-producing pancreatic β -cell. In type 1 diabetes, it is the autoimmune attack on the β -cells that leads to the complete loss of insulin production, which necessitates insulin therapy for survival. Also in obesity-driven “classical” type 2 diabetes, it is the failure of the β -cell to cope with the increased demands that precipitates increased blood glucose levels and onset of the disease [115]. In addition to this, several insulinopenic types of type 2 diabetes are described, in which restricted insulin production and secretion appear as the main pathogenic factor. Such insulinopenic type 2 diabetes variants include monogenic maturity-onset diabetes in the young (MODY1-6) [121].

Increased prevalence of obesity has been identified as the main environmental factor causing the worldwide explosion in the incidence of type 2 diabetes. However, it is also well established that type 2 diabetes exhibits strong inheritance. The identification of the molecular genetics of type 2 diabetes started in the early 1990s and turned out a most challenging task. The advent of the HapMap and novel high-throughput technologies enabled the development of genome-wide association scans (GWAS), which represent one of the main milestones in modern medical research. Such studies have enabled identification of a number (around 30 to date) of common genetic variants (i.e. single-nucleotide polymorphisms, SNPs) that associate with type 2 diabetes [38, 98, 103, 128]. In agreement with this, some of the diabetes-associated SNPs correlate with an increased body weight, for instance in the fat mass and obesity-associated (FTO) gene [28]. However, for many it came as a surprise that the vast majority of genetic variations in type 2 diabetes are related to a reduced capacity for insulin secretion [39]. At present we largely lack exact knowledge about how inherited genetic variations result in β -cell dysfunction in individuals that develop type 2 diabetes. Apart from the mutations causing monogenic MODY forms that also play a modest role in the development of common type 2 diabetes, this has so far only been convincingly demonstrated for the SNP rs553668 in the ADRA2A gene that is associated with increased expression of the inhibitory adrenergic α -2A receptor in pancreatic β -cells, resulting in impairments in the insulin release machinery and reduced insulin output in response to glucose [96]. This is a typical example of a “functional” defect, but inherited malfunctions are also suggested to lead to reductions in the number, or mass, of insulin-producing β -cells (collectively referred to as “ β -cell mass”), producing a situation similar phenotype, albeit less dramatic, as in autoimmune destruction in type 1 diabetes. In fact, several reports suggest that β -cell mass is 30–40% reduced in type 2 diabetes, but the exact implications of these findings is still a matter of

debate [18, 40, 97, 88]. Be that as it may, the central role of the β -cell in any type of diabetes mellitus is today generally accepted. In the future, important tasks in diabetes research are to pinpoint the molecular defects associated with diabetes and to develop strategies to correct them. In the light of this, it is timely to comprehensively review of the molecular reactions that contribute to the important overall functions of the β -cell.

2.2 The Basic Organization and Characteristics of the Exocytotic System in Pancreatic β -Cells

2.2.1 *Synthesis of Insulin and Formation of Insulin Granules*

The main role of the pancreatic β -cell is to control blood glucose concentrations in the body. This it does by production and storage of the glucose-lowering peptide hormone insulin in secretory granules, followed by their subsequent release into the blood stream by regulated exocytosis whenever blood-glucose levels tend to increase above the set value ~ 5 mM. Insulin is formed in the endoplasmic reticulum (ER) in its precursor form proinsulin, which is later converted by a series of peptidase cleavage by prehormone convertases 1 and 3 (PC2 and PC1/3, respectively) into mature insulin [7, 106]. These changes start already in the Golgi apparatus, where the insulin granules are formed by budding. Transport of membranes (i.e. granule precursors) in the ER to the Golgi and further transport of formed granules to the plasma membrane is controlled by a family of small regulatory GTP-binding Rab proteins [29, 107]. These are likely to play similar roles in the β -cell, but their exact actions in insulin secretion remain largely unexplored. The secretory granules are at this stage in their immature form, which in electron microscopy (EM) is characterized by an opaque appearance of insulin [79]. Maturation of insulin granules is visible as a condensation of insulin into a dense core, and formation of insulin crystals. This process occurs in parallel with a marked acidification of the insulin granule interior. Estimates of pH in the ER and *cis*-Golgi complex are typically close to the overall 7.2 in the cytoplasm, whereas the granule interior becomes increasingly acidic and in the mature granule is down to pH 5 [51, 53, 80] (Fig. 2.1).

The generation of the acidic milieu of the insulin granule is an active energy-consuming process driven by the v-type H^+ -ATPase [52]. However, counter-ion fluxes over the granule membrane exert a permissive function in this process and in their absence proton translocation over the granule membrane would quickly be counteracted by the build-up of a strong positive granule membrane potential [9, 61, 112]. This process is — at least in part — mediated by the chloride granule transporter/ion channel CIC-3 that localizes to the granule membrane and is required for glucose-stimulated insulin secretion [62].

Granule acidification is necessary for a well-functioning insulin secretion apparatus. First, it is necessary for allowing insulin processing and achieving the acidic

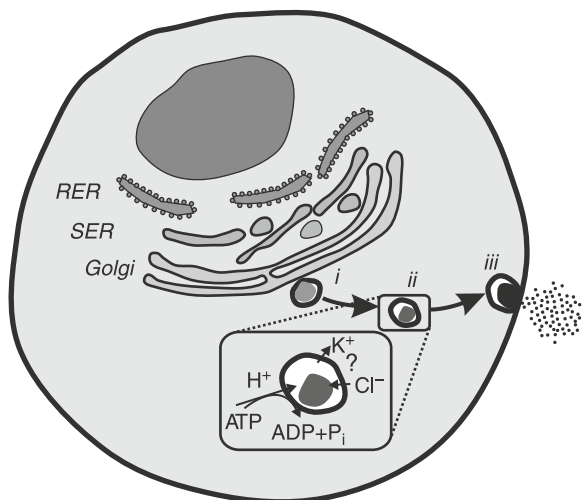


Fig. 2.1 Formation and maturation of the dense-core insulin granule. Insulin is synthesized in the rough endoplasmic reticulum (RER) and further processed in the smooth ER (SER) and the Golgi network from which immature insulin granules bud off (i). After exit from the Golgi apparatus, the granule interior has a pH ~ 7.2 and is acidified by the action of the v-type ATPase, a reaction that is facilitated by counter-ion fluxes (ii). Acidification is necessary for proinsulin processing and condensation of the insulin core, which is a hallmark of the mature and releasable insulin granule (iii).

pH-optimum of PC3 that cuts off the C-peptide to form the mature insulin molecule [7]. Second, an acidic granule interior is essential for the insulin granules to become, and stay, releasable [12]. This and other aspects of the functional organization of the insulin release machinery are further developed in Section 2.2.2.

2.2.2 Insulin Granule Transport to Release Sites

The importance of the cytoskeleton for intracellular transport of insulin granules was established in a series of landmark electron microscopy studies by Orci and co-workers [78, 117]. These studies demonstrated that the microtubule system is essential for transporting insulin granules from the *trans*-Golgi network in the cell interior to the release sites at the plasma membrane. These findings were corroborated by physiological studies of insulin secretion, demonstrating that destruction of the microtubule system in islets using inhibitors such as colchicine and vincristine led to suppressed glucose-stimulated insulin secretion [117]. These early observations on the role of the cytoskeleton for insulin granule transport and release were made using electron microscopy and could only provide snapshots of insulin granule location at different time points and did not possess the time resolution sufficient for tracking and characterizing the insulin granule motion pattern. A first step towards achieving this was made using high-speed phase-contrast imaging in monolayers of

foetal rat islet cells [59]. However, this field of research did not boom until visualizing specific proteins became possible by construction of fluorescently labelled chimeric proteins using genetically encoded green fluorescent protein (GFP) and its derivatives [84]. In conjunction with increased availability of confocal and other high-speed imaging techniques, protein trafficking studies for the first time became feasible.

Typical Length Scales (Rough Estimates of Diameters) in β -Cell Research

- 1 Å = 0.1 nm water molecule, cations (Na^+ , K^+ , Ca^{2+} , Mg^{2+} etc.)
- 2 nm cross section of DNA string
- 7 nm lipid bilayer plasma membrane
- 10 nm insulin crystals, proteins, fluorescent dyes, quantum dots
- 30 nm virus
- 100 nm = 0.1 μ insulin granules, magnetic beads
- 5 μ nucleus
- 10 μ β -cell
- 100 μ = 0.1 mm Langerhans island
- 20 mm = 2 cm cross section of pancreas

Further Reading:

Alberts B et al (2002) *Molecular biology of the cell*, 4th edn. Garland Science, New York, NY

Added by the editors

For the study of insulin granule movement using GFP-derived fluorophores, such as enhanced green fluorescent protein (EGFP), the most straightforward idea would be to couple the EGFP directly to insulin. For most groups this turned out a cumbersome approach, because of trapping of the chimeric EGFP-insulin protein in the ER, but Nagamatsu and colleagues were more successful and have contributed to the field with a series of important papers [70, 75–77] that literally illuminated the phasic nature of single-cell exocytosis in β -cells. Alternative approaches employed by other groups include fluorophore tagging of granule transmembrane proteins such as phogrin or the exocytotic vesicle-associated membrane protein 2 (VAMP2) [68, 87, 113, 114], which enabled the first direct investigations of different modes of exocytosis: so-called kiss-and-run exocytosis in which the granule remains more or less intact for recycling after transiently fuses with the cell membrane, as opposed to full exocytotic fusion when the granule lipid bilayer is fully incorporated in the cell membrane. A series of important papers have also fluorescently labelled insulin granule cargo protein insulin such as insulin amyloid polypeptide (IAPP) that is synthesized by the β -cell and stored and co-secreted with insulin. These studies have thoroughly investigated the relative contribution of kiss-and-run and full exocytosis and have pointed out that the exocytotic process is not an all-or-none process, but that regulation of the width of the fusion pore offers the flexibility to allow

release of smaller granule constituents, like ATP, without release of the bulkier insulin molecule [10, 11, 74].

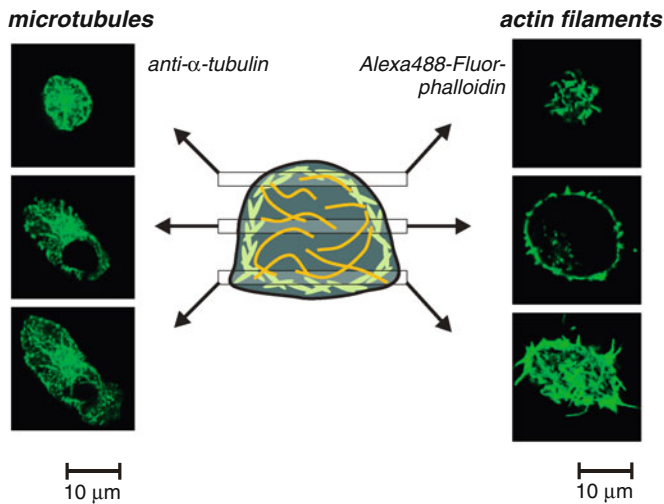
These advances were also facilitated by the development of imaging techniques such as high-speed imaging by confocal spinning disc technology or evanescent wave/total internal reflection (TIRF) imaging produced a great leap forward in our understanding of the pre-exocytotic events in insulin secretion [11, 68, 74, 114]. With confocal imaging the width of the focal plane can be set to any layer of the cell, whereas with TIRF imaging it is only possible to illuminate the ~ 100 nm closest to the part of plasma membrane attached to the bottom of the cell culture dish. This imposes a certain limitation, but is also a blessing as it makes it possible to image the events occurring at the plasma membrane, e.g. exocytosis, with high temporal resolution and under optical conditions superior to what can be achieved by confocal imaging for the same type of investigations.

2.2.2.1 Directed and Random Granule Movement

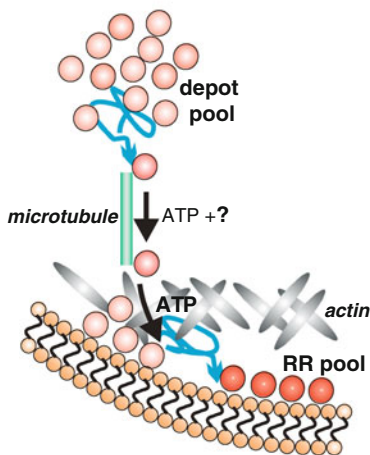
Studies of granule translocations in the cytosol have primarily been studied by confocal imaging combined with tracking of individual granules by specialized software. Such studies using EGFP-phogrin or EGFP-IAPP have demonstrated that insulin granules exhibit extensive mobility already in the resting state, i.e. under low-glucose conditions. The granule transport activity by far exceeds that necessary for merely transporting the insulin granules from the site of formation by budding off the *trans*-Golgi network and the few micrometres to the plasma membrane. The granule motion pattern is a mixture of directed transport events and random movements. Both type of events occur throughout the entire cell volume (save for the nucleus) and can sometimes be observed in sequence [2, 54].

Random movement can be quantified and distinguished from the directed events by analysis of the granule trajectories obtained by the tracking software. The mean squared displacement is calculated for given time periods, which in this type of study typically range from that between two consecutive images and up to 10 s. Plotting the MSD value versus that of the length of this time period will identify granule motion as being restricted, random (diffusional) or directed. The restricted events are those where the granule experiences some type of hindrance, e.g. the cytoskeleton or other parts of the cytoarchitecture, which in the MSD plot is represented by MSD values reaching a plateau within few seconds. Diffusional events describe a straight line in the MSD plot, from which the diffusion constant D can be calculated, whereas directed events are best fitted to second degree equations. Directed events can cover several micrometres in just a few seconds, whereas granule translocation by random is 10- to 100-fold slower process. In fact, if one extends the length of time periods studied to e.g. 1 min, it is evident that granule movement is overall restricted in the β -cell. By this type of analysis it was estimate that the average granule can diffuse freely within functional “cages” of ~ 0.9 μm diameter. Cytoskeletal elements form at least partly a physical barrier for random granule movement and disruption of the microtubule system increased the average limit for free granule diffusion by $\sim 30\%$ (Fig. 2.2).

A



B



C

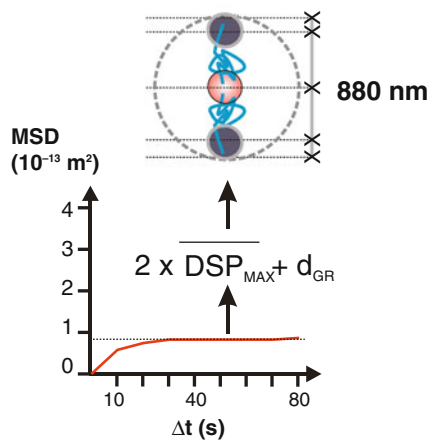


Fig. 2.2 Cytoskeleton and insulin granule motility. (a) In the β -cell microtubules are found throughout the entire cell volume, whereas actin filaments primarily form a cortex just beneath the plasma membrane. Example stainings are from a clonal insulin-secreting INS-1 cell. (b) Directed and random insulin granule translocations are observed throughout the cell; often in sequence when tracking an individual granule. Directed movement occurs along cytoskeletal elements, whereas diffusional random movement seems to be of particular importance during changes in transport system. Both types of motion are essential for refilling the readily releasable (RR) pool of insulin granules. (c) The average insulin granule can diffuse freely within a functional cage of 880 nm. This value was obtained by adding the experimentally observed double average maximal displacement (DSP_{MAX}) by diffusion for single insulin granules to the average granule diameter (d_{GR}) (cf [54]).

2.2.2.2 Cytoskeleton and Motor Proteins in the β -Cell

Cargo transport along microtubules is driven by kinesin motor proteins. The human genome contains 25 gene families. Kinesins typically pair to form dimers consisting of two heavy chains and two light chains. The heavy chain contains the motor domain in the globular head, which is connected via a short, but flexible, neck linker region to the long central coiled-coil stalk, ending in the tail region that associates with a light chain. The signature of all kinesin variants is the head region, the amino acid sequence of which is highly conserved. Both ATP binding, hydrolysis and ADP release affect the conformation of the microtubule-binding domains and the position of the neck linker relative to the head. The resulting molecular twisting movement is what generates motion in the kinesin molecule, probably by a “hand-over-hand” mechanism in which the head regions of the kinesin dimer alternate in the leading position. Nearly all kinesin isoforms mediate transport from the cell interior to the periphery (antegrade transport), but the kinesin-14 family and the entire dynein motor family drive transport in the opposite retrograde direction. In the β -cell, there is good evidence for a central role of conventional kinesin-1 in antegrade insulin granule transport in the microtubule system and insulin. This was first suggested by experiments using an antisense approach to suppress kinesin-1 expression [67] and later convincingly demonstrated by studies using dominant-negative kinesin-1 [118, 119].

In the β -cell the microtubules are found throughout the entire cell volume, but in the cell periphery actin filaments form a tight network [78, 54, 55, 120]. The main function of this actin cortex is to introduce a bottleneck in insulin secretion and to provide a physical barrier preventing granule diffusion to the release sites uncontrolled release of insulin. Breakdown of the actin network strongly accelerates insulin release in single cells, as well as in intact islets. The actin filaments also conduct cargo transport generated by the action of myosin motor proteins. The myosin superfamily family contains 17 classes of molecular motors. The myosin superfamily is represented in virtually all eukaryotic cells, and each cell type typically contains a set of different myosin variants. In the β -cell, there is evidence for the expression and function of the motor myosin Va that transports granules in the antegrade direction [54, 120], whereas myosin VI is involved in retrieval of cell membrane in the endocytic pathway [17]. Expression of additional myosins, e.g. myosin 1c, has also been reported, but their actions remain unestablished. Myosin Va appears not to drive long-ranging granule translocations in the β -cell, since downregulation of the protein does not result in a noticeable decrease in granule trafficking. Rather, the motor protein acts a gatekeeper, controlling granule supply to the release sites at the plasma membrane. The interaction with the insulin granule is likely to involve the small G-protein Rab27a and its interaction partner Slac-2c/MYRIP [30]. Slac-2c/MYRIP has been reported to reversibly interact with both the insulin granule and actin, suggesting that this regulatory molecule can block, or permit, myosin-5a-driven granule transport along the actin cortex [123]. Ultimately, the insulin granule detaches from both myosin-5a and Slac-2c/MYRIP at the peripheral face of the actin cortex and presumably covers the remaining 10–100 nm to the plasma membrane by diffusion [54].

2.2.3 Functional Insulin Granule Pools and the Relation to Phasic Insulin Secretion

Several studies have used TIRF imaging of EGFP-tagged insulin to investigate exocytosis of insulin granules during glucose-stimulated phasic insulin secretion [69, 75, 101]. These studies have provided proof in real time for earlier observations using immunoprecipitation techniques demonstrated that during early (first) phase insulin secretion is primarily due to the release of insulin granules already docked at the plasma membrane (resident granules), whereas during subsequent late phase secretion insulin granules are recruited from the cell interior (newcomer granules) [21].

These findings are the natural continuation of functional studies of single-cell exocytosis made during the 90 s using either membrane capacitance measurements or carbon fibre amperometry [26, 90, 91, 95]. These studies demonstrated a striking resemblance of the properties of exocytosis in the single β -cell and that of insulin secretion in vivo: after stimulation a rapid initial component of insulin release is seen in both single cells and in vivo, which is followed by release at lower rates. This typical biphasic pattern of insulin secretion is a well-known phenomenon in vivo, as well as in isolated islets, and was first described in the 60s [34, 35]. After a glucose load, first-phase insulin secretion characterized by high rates of insulin secretion lasts for 5–10 min, thereafter follows a temporary low in insulin release (nadir phase), before the second phase starts during which insulin secretion increases to reach a plateau where it remains for hours. The underlying mechanisms long remained unresolved, but pioneering modelling work by Cerasi and Grodsky established a bi-compartmental model [32, 33]. The reason why phasic insulin secretion has remained a topical issue is because the first sign of imminent diabetes is the loss of first-phase insulin secretion already in the pre-diabetic state [23, 24, 33, 35]. To add a further level of complexity to the picture, insulin release is also of pulsatile or oscillatory in nature, similar to release of other hormones, such as growth hormone. Phasic insulin secretion as measured in the circulation or in secretion assays with a time-base in the min range represents an integral of peaks of insulin secretion. The oscillatory nature is not confined to the final release event, but also applies to glucose metabolism, intracellular signal transduction and electrical activity and represents a vast topic on its own [45, 109]. The importance of these endeavours is underpinned by the fact that both the amplitude and the frequency of the insulin secretion peaks are affected in pre-diabetes [86].

Phasic insulin secretion remains a useful model as a foundation for better understanding the insulin release machinery in health and disease. Interestingly, first-phase secretion of insulin secretion can be evoked by agents with a merely depolarizing action, such as high extracellular K^+ , or K_{ATP} channel blocking sulphonylureas, whereas sustained release requires metabolic fuel; in the single cell in the form of supply of Mg-ATP and in the whole body in the form of glucose [26, 44]. Further experimental support for this idea was provided by data from experiments in isolated rat islets elaborating on the temperature dependence of insulin secretion. Performing the experiments at room temperature (24°C) rather than body

Observation Means and Scales – From Light Microscopy to Electron Microscopy

Throughout history, biologists and medical doctors have applied various systems to view samples or objects:

The *human eye* is a natural optical system. For the normal eye, the minimal resolution at the minimal distance $D = 25$ cm of distinct vision is approximately 0.08 mm.

Light microscopy was discovered by craftsmen making eyeglasses as early as the sixteenth century in the Netherlands and Northern Italy. In 1609–1610, Galilei used the optic tube he had designed as a microscope. Around 1665, R. Hooke established the cellular structure of animal and plant tissues. In 1872–1873, E. Abbé developed the now classic theory of image formation with non-self-luminous objects by passing visible light transmitted through or reflected from the sample through a single or multiple lenses to allow a magnified view of the sample. The modern microscope can distinguish structures with only $0.20\ \mu$ between elements.

Fluorescence microscopy is extremely powerful due to its ability to show specifically labelled structures within a complex environment and also because of its inherent ability to provide three-dimensional information of biological structures.

Quantum dots have been found to be superior to traditional organic dyes on several counts, one of the most obvious being brightness as well as their stability (allowing much less photobleaching). Cadmium-free quantum dots are being developed.

Confocal microscopy generates the image by using a scanning point of light instead of full sample illumination. It gives a slightly higher resolution, and significant improvements in optical sectioning by blocking the influence of out-of-focus light which would otherwise degrade the image.

Magnetic resonance imaging (MRI) is a non-invasive imaging modality. In addition to conventional MRI of anatomical structure and function, recent advances have led to the development and application of MRI for targeted molecular and cellular imaging.

Electron microscopy (EM) has been developed since the 1930s. It uses electron beams instead of light. Because of the much smaller wavelength of the electron beam, resolution is far higher ($0.5\ \text{\AA}$ in 2010). EM requires the fixing of the object by rapid freezing and is therefore not immediately applicable for tracing dynamics in vivo.

X-ray microscopy has also been developed since the late 1940s. The resolution of X-ray microscopy lies between that of light microscopy and the electron microscopy.

Scanning probe microscopes like the atomic force microscope (AFM) have special requirements for the shape of the probe. Interaction with the probe can not always be avoided and is sometimes wanted.

Further Reading:

Douglas B (2008) Murphy. Fundamentals of light microscopy and electronic imaging, 2nd revised edn. Wiley, New York, NY

Added by the editors

temperature (37°C) has little effect on first phase, but strongly suppresses second phase [6, 14]. In agreement of this finding, insulin secretion evoked by high K^+ is affected to a lesser degree by reductions in temperature than is glucose-evoked secretion [22]. Studies in isolated mouse β -cells revealed a similar temperature dependence of exocytosis that primarily affects late components of exocytosis that require Mg-ATP to occur [90] (Fig. 2.3).

In the single cell, the granules released during the rapid initial component of exocytosis are referred to as the readily releasable pool (RRP). These are standby granules waiting for an increase in intracellular Ca^{2+} [Ca^{2+}]_i, the cue for regulated exocytosis. Granules cannot be released until they are primed for exocytosis, which is a reaction that involves hydrolysis of Mg-ATP [26, 48, 83]. The RRP granules have undergone this preparation and can exocytose immediately upon elevation of [Ca^{2+}]_i. For continued exocytosis to occur, new granules must be recruited from a reserve pool and undergo Mg-ATP-dependent priming. These reactions are collectively referred to as mobilization. The RRP in single mouse- β -cells has been estimated to contain ~50–70 granules [26, 122]. Using this value with the insulin content of the average insulin granule (~2 fg), one can compare it with the amount

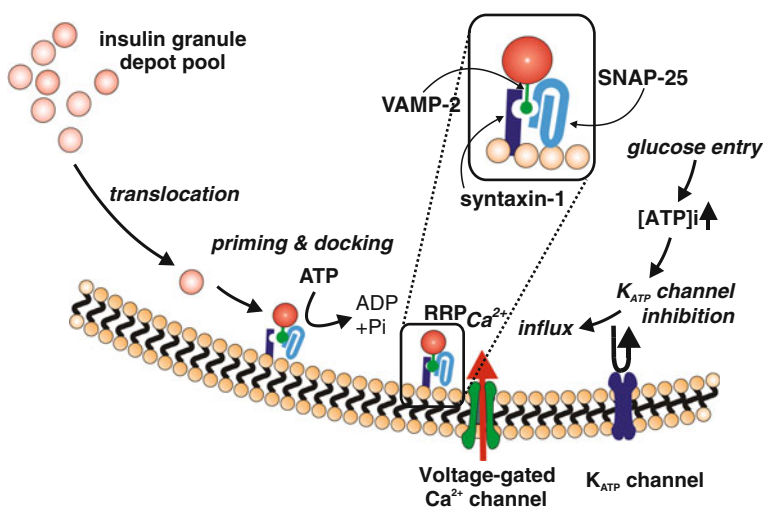


Fig. 2.3 Functional insulin granule pools. Insulin granules are recruited from a reserve depot by physical translocation to, and docking at, the cell membrane. Coinciding with this process an ATP-requiring biochemical modification takes place and is coined priming. Granule translocation and priming are collectively referred to as mobilization. Docking at the cell membrane involves formation of the exocytotic core complex involving the transmembrane SNARE-protein syntaxin-1, the membrane-associated SNAP-25 and the granule protein VAMP-2 (*inset*). Primed and docked insulin granules form the RRP of insulin granules that is released instantaneously upon increases in cytoplasmic Ca^{2+} , the cue for regulated exocytosis. Activation of voltage-gated Ca^{2+} channels is the end result of glucose-stimulated generation of electrical activity that involves the membrane potential-regulating ATP-regulated K^+ channels (K_{ATP} channels).

of insulin released from an average mouse islet containing 1000 β -cells. Such exercises reveal a good correlation between the amounts of insulin released per cell during first phase and the RRP. This and accumulating reports [57, 99] support to the idea that release of RRP is the cellular correlate to first-phase insulin secretion.

2.2.3.1 Role of Cytoskeleton in Phasic Insulin Release

The exact nature of the priming reaction remains unclear; it was previously believed that priming was accounted for by the ATP-ase activity of the NSF (*N*-ethylmaleimide-sensitive factor) [8, 43, 81], but this enzyme is currently regarded to be responsible for break-up of the exocytotic protein core complex during membrane retrieval following exocytosis [13, 105]. It is possibly more correct to regard priming as a term that collectively describes a number of ATP-dependent reactions that are required for exocytosis to occur. Candidate priming reactions include directed insulin granule transport driven by motor proteins along the cytoskeleton. The observation that inhibition of kinesin-1 using a dominant-negative approach selectively suppressed late phase secretion is suggestive of a priming action of this molecular motor in exocytosis [118]. However, single-cell studies of exocytosis in cells treated with microtubule inhibitors demonstrated that although exocytotic rates were reduced, the characteristic biphasic release pattern remained intact [54]. These findings suggest that the transport activity of the microtubule system should rather be regarded as the “volume control” of the β -cell, but not being particularly important for the “quality control” that shapes insulin secretion and ultimately regulates the supply of insulin granules to the RRP at the plasma membrane. Instead, the interactions between actin-myosin in the cell periphery appear as the more likely priming reaction candidates. The first general argument in favour of this idea would be these reactions occur closer (temporally as well as spatially) to the final release event, but is also backed by experimental data using TIRFM imaging showing that a reduced insulin granule transport to the plasma membrane becomes apparent only during second phase in myosin-5a-silenced cells [54]. Be that as it may, compelling evidence for the involvement of motor proteins in granule priming is missing, but their involvement in granule mobilization is firmly established.

2.2.3.2 Exocytosis-Regulating Proteins of the β -Cell

Insulin granule release is mediated by regulated Ca^{2+} -dependent exocytosis. Exocytosis is a tightly regulated process in all excitable secretory cells and the β -cell is no exception. A large number of proteins are in one way or another involved in controlling or modulating exocytosis, but the centre stage is taken by the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein family [41, 42, 81, 105]. These complex-forming proteins are of undisputed importance for exocytosis in eukaryotic cells, but the exact function in membrane fusion remains controversial. In the β -cell, the exocytotic core complex consists of two t-SNARE proteins SNAP-25 (synaptosome-associated protein of 25 kDa) and syntaxin 1A that locate to the target membrane, i.e. the plasma membrane,

and their binding partner in the insulin granule membrane, the v-SNARE protein VAMP2 (vesicle-associated membrane protein 2 aka synaptobrevin2). An alternative classification of the SNARE proteins is based on the presence of arginine or glutamine residues in the core domain of the SNARE protein, coining the proteins as either R- or Q-SNARES [27]. Most v-SNARE proteins are thus classified as R-SNARES, and the fusion competent core complex contains four-helix bundles consisting of three Q-SNARES (one from syntaxin 1A and two from SNAP-25), and one R-SNARE from VAMP2.

In the β -cell, the Q-SNARES reside on the plasma membrane side. Syntaxin 1A is a transmembrane protein with the membrane spanning domain located in the carboxy-terminal region and contains one SNARE core domain. The membrane-associated SNAP-25 is coupled to the plasma membrane via four palmitoylated cysteine residues in the central linker domain and has two SNARE core domains. The R-SNARE VAMP2 has a carboxy-terminal transmembrane domain and one SNARE core domain. All three proteins interact in the SNARE core complex in which the amino terminus of SNAP-25 binds to syntaxin 1A, whereas the carboxy-terminal binds to VAMP2. This leads to the formation of the four-helical bundle that is believed to pull the vesicular membrane onto the plasma membrane and leads to fusion of the two membranes [108].

2.2.3.3 Voltage-Gated Calcium Ion Channels (Ca_V) in the β -Cell

Excitable cells such as the β -cell possess ion channels that are sensitive to specific changes in the environment or in neighbouring cells. This leads to the generation of electrical signals in the form of fluctuations in the membrane potential. Taking the β -cell as an example, elevations in the circulating blood glucose concentrations result in increased glucose uptake into the β -cell via the glucose transporter (GLUT). The sugar is then rapidly phosphorylated in a rate-limiting reaction by glucokinase that controls the entry to β -cell glucose metabolism via glycolysis and mitochondrial metabolism resulting finally in increased intracellular ATP concentrations, which is the topic of [Chapter 3](#) of this volume (Fig. 2.4).

The changes in the β -cell metabolic status upon blood glucose elevations are sensed by the ATP-sensitive potassium channels (K_{ATP} channels, reviewed in [5]). Under low-glucose conditions, these channels conduct a tonic outward flux of positively charged potassium ions (K^+), which maintains a negative (-70 mV) membrane potential and puts the β -cell at rest. When ATP levels increase, the K_{ATP} channels close and positive charges accumulate inside the β -cell leading to a slow depolarization of the β -cell to the threshold potential (~ -40 mV), where voltage-gated ion channels are activated (opened) and generate action potentials by influx of positive ions. In mouse β -cells, action potentials are generated by voltage-gated calcium channels (Ca_V channels) only, whereas in rat and human β -cells the depolarizing action of voltage-activated sodium channels (Na_V channels) are necessary for activation of the Ca_V channels [15]. The influx of Ca^{2+} ions leads to an elevation in the cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which is the trigger

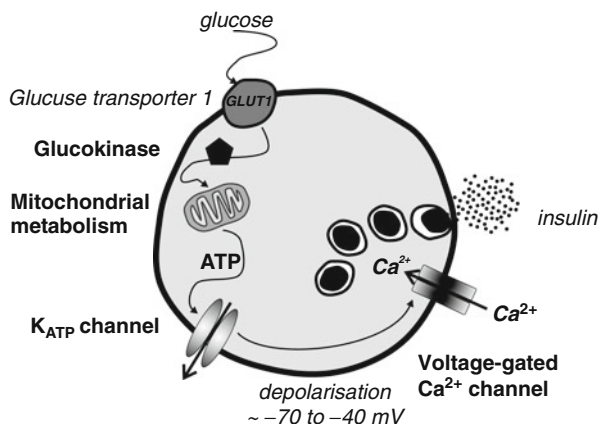


Fig. 2.4 The stimulus-secretion coupling of glucose-evoked insulin secretion. Insulin secretion by regulated exocytosis is the end result of the chain of events that start with increases in plasma glucose and uptake of the sugar into the cell via glucose transporters (GLUT1 in human β -cells and GLUT2 in rodent). Inside the cell glucose is immediately phosphorylated by glucokinase and enters glycolysis and mitochondrial metabolism to yield an increase in ATP. This inactivates the membrane potential-regulating ATP-regulated K⁺ channels (K_{ATP} channels), which depolarizes the β -cell membrane potential from the resting -70 mV to the threshold potential (~ -40 mV), at which the voltage-gated Ca²⁺ channels activate. This leads to the generation of Ca²⁺-dependent action potentials that trigger insulin release. Not illustrated here are the actions of mitochondrial glucose metabolites (e.g. glutamate, NADPH) that amplify Ca²⁺-regulated exocytosis.

signal for regulated exocytosis of the insulin-containing secretory granules. An additional K_{ATP}-independent action of glucose on Ca²⁺-evoked insulin secretion, coined amplifying action by Henquin [44], has been reported from several laboratories. This action involves a product/products of glucose metabolism, the exact identity of which is not unequivocally established, but the mitochondrial metabolite glutamate [65] and the reducing equivalent NADPH [56] have both been suggested. This topic is covered in fuller detail in [Chapter 3](#) of this volume.

Elevations in [Ca²⁺]_i are important signals in many biochemical pathways in the cell, which control β -cell function and survival. Taking the mouse β -cell as an example, insulin release is triggered by activation of voltage-gated Ca²⁺ channels (Cav). There are ten different isoforms of Cav channels, of which the β -cell has been reported to express mRNA transcripts for Cav1.2, Cav1.3, Cav2.1, Cav2.2, Cav2.3 and Cav3.1 [126]. It remains unclear whether all these channels are expressed on the protein level and whether they are functional. For instance, transcripts for both L-type channels Cav1.2 and Cav1.3 are present in islets, but mouse knockout studies clearly suggest that it is Cav1.2 that is the functionally important one in adult cells [99, 102, 125], although conflicting reports exist [126, 127]. However, Cav1.3 is important for β -cell expansion and affects β -cell mass [71], suggesting that its major role is during embryonal and early postnatal development. In addition, β -cells also express R-type Cav2.3 channels. The factors that determine the

exact action of the calcium ion include the amplitude and duration of the $[Ca^{2+}]_i$ increase, but also the exact site of $[Ca^{2+}]_i$ elevation in the cell. For example, whereas L-type $Ca_v1.2$ channels couple to the exocytotic machinery and trigger release of the rapid component of exocytosis coined the RRP, whereas R-type $Ca_v2.3$ channels are more important for a late component of exocytosis. Similarly, in the *in vivo* situation $Ca_v1.2$ is associated with rapid first-phase insulin secretion [99], whereas R-type $Ca_v2.3$ is coupled to the sustained second phase [57]. The different actions of the different Ca_v isoforms equips the β -cell with the means to fine-tune the secretory response and cell signals that specifically alter the activity of a Ca_v channel isoform may therefore affect the kinetics of insulin secretion (Fig. 2.5).

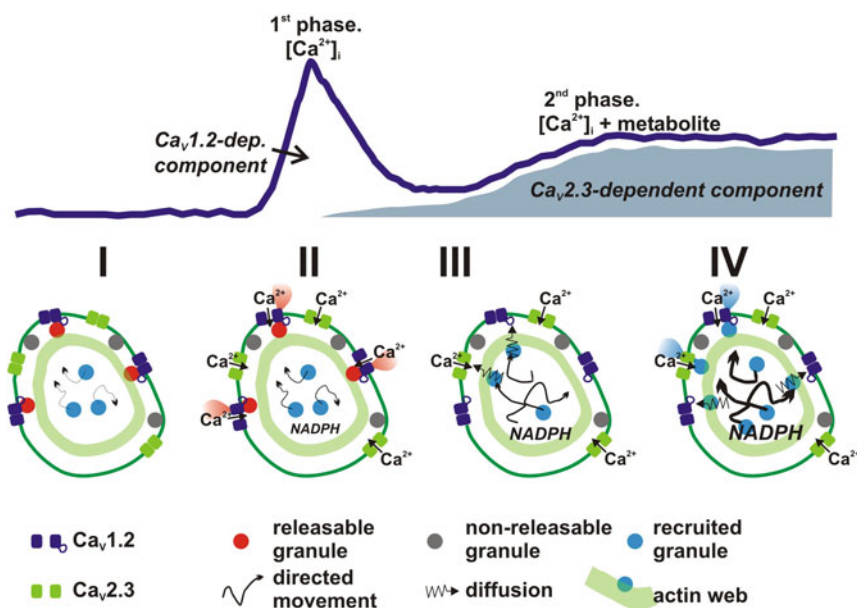


Fig. 2.5 Voltage-gated Ca^{2+} channel (Ca_v) activation and insulin granule movement during phasic insulin secretion. In the prestimulatory phase (I), central insulin granule movement along microtubules is ongoing, whereas primed insulin granules in the readily releasable pool are docked at the plasma membrane and associated with L-type $Ca_v1.2$ channels. Upon stimulation with glucose (II), insulin granules close to $Ca_v1.2$ are exocytosed during first phase secretion and cytosolic NADPH increases. During the nadir phase (III), insulin release rates temporarily decrease when the RRP has been emptied, meanwhile NADPH continues to increase and insulin granule mobilization by directed and random movement is accelerating. During second phase insulin secretion (IV), a new steady state has been achieved and NADPH reaches maximal levels. Insulin granule mobilization is now rate limiting for secretion rates and R-type $Ca_v2.3$ channels are now more important for insulin secretion. Note that NADPH is given as one example of a metabolite that can affect late-phase insulin secretion, but other metabolites have also been suggested to fulfil the same action. During the whole process, certain granules docked at the plasma membrane remain un-released and probably reflect defect insulin granules that are un-primed and destined for degradation.

2.2.3.4 Hormonal Modulation of Glucose-Evoked Insulin Secretion

Both glucose-evoked Ca^{2+} signalling and the efficacy of the Ca^{2+} -responsive exocytotic machinery are modulated by hormones and neurotransmitters. These can be grouped in different ways; one is to simply label them as enhancers, or inhibitors, of insulin secretion. Enhancers of insulin secretion include hormones that increase cytoplasmic levels of cyclic AMP [3, 19, 58]. This all important second messenger in insulin secretion activates the cyclic AMP-regulated kinase or protein kinase A (PKA). PKA changes the function of proteins by reversibly phosphorylating serine or threonine amino acids in specific sites. However, exactly which proteins are available for phosphorylation is different among cell types, because protein composition varies from cell type to cell type. PKA appears to activate the exocytotic machinery broadly [3, 91]. For example, cAMP promotes granule translocation and PKA indeed phosphorylates proteins such as synapsin-1 believed to be involved in such upstream cellular processes in the exocytotic chain [49]. PKA also phosphorylates SNAP-25 of the exocytotic core complex which indicates that PKA may regulate size of the readily releasable pool [46]. Cyclic AMP also has PKA-independent actions [91] which are mediated by the sensor protein EPAC2 [82]. This parallel and equally important mechanism seems to act on granules that are about to be released, i.e. in the readily releasable pool. The exact action of EPAC2 in the β -cell is not fully elucidated, but an action on the intragranular ion homeostasis and an effect on granular pH has been suggested [28]. Collectively, these cAMP-sensing systems are of fundamental importance for insulin release and increase the efficacy of Ca^{2+} -dependent exocytosis of insulin up to 10-fold.

Among cAMP-elevating hormones that belong to this group are glucagon that is released from the islet α -cells, gastric inhibitory peptide, aka glucose-dependent insulinotropic peptide (GIP), and not least the glucagon-like peptide 1 (GLP-1). GLP-1 and GIP are the main so-called incretin hormones, which refers to the fact that both released upon food ingestion from the gastrointestinal tract and act as insulin secretagogues [36, 37, 110, 111]. In particular GLP-1 has several advantageous effects on blood glucose homeostasis and this signalling system is used clinically in the treatment of type 2 diabetes [47, 66, 85]. Using the peptide as such for treatment is not feasible because of the rapid breakdown of the biologically active variant GLP-1 (7–36) amide, which necessitates continuous infusion. To this end several long-lasting GLP-1 analogues have been developed, such as exenatide or liraglutide. However, by far the most important approach clinically is to inhibit the enzyme that cleaves and inactivates GLP-1 and GIP, dipeptidyl peptidase-4 (DPP-4) [20, 31]. This treatment can be given orally (as tablets) and several such DPP-4 inhibitors have been developed, e.g. sitagliptin.

Other enhancers of insulin secretion, include acetylcholine, released from pancreatic parasympathetic nerve endings, or cholecystokinin, which both act via G-protein-coupled receptors that activate phospholipase C, resulting in formation of inositoltrisphosphate (IP_3) that has the capacity to liberate Ca^{2+} ions from internal stores, but also generation of diacylglycerol that activates protein kinase C enzymes of the conventional and novel subtypes. These signalling proteins are well-established enhancers of insulin release that act by reversible phosphorylation of

hydroxyl groups on serine and threonine amino acid residues in target proteins; example target proteins include presynaptic and SNARE-interacting munc proteins [63] and CAPS [73]. The net result is an augmentation of glucose-evoked insulin release, by directly elevating the cytoplasmic concentration of Ca^{2+} ions, as well as an increased efficacy of the proteins in the exocytotic machinery.

The classical inhibitors of insulin secretion include adrenaline (exposed to the islet as a neurotransmitter from sympathetic nerve endings or as a hormone via the blood flow), the peptide hormone somatostatin released from the islet delta-cells and the peptidergic neurotransmitter galanin [1, 72, 89, 93, 94, 116]. All these insulin suppressors act via receptors coupled to heterotrimeric GTP-binding proteins that contain the inhibitory Gi/Go subunits that directly suppress the exocytotic machinery [60]. In fact, these inhibitors suppress insulin release in the β -cell by several

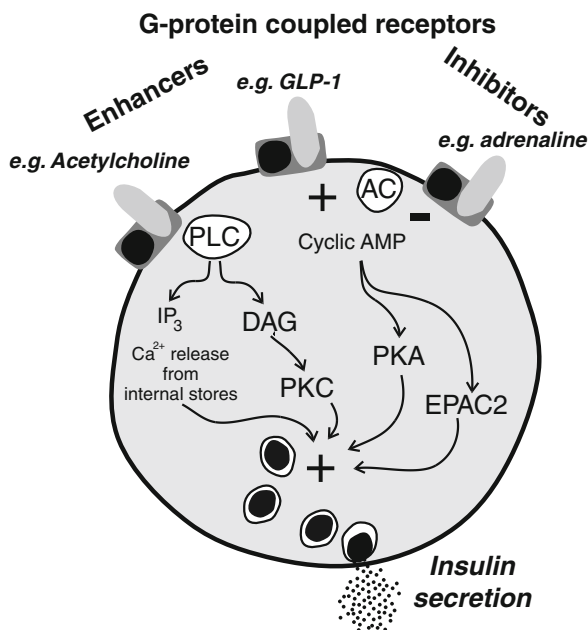


Fig. 2.6 Modulation of insulin secretion by hormones or neurotransmitters. Hormones or neurotransmitters that stimulate insulin secretion, either by themselves or in the presence of glucose, are named enhancers. Examples are acetylcholine that activates phospholipase C (PLC) that generates inositoltrisphosphate (IP_3) that stimulate insulin secretion independently of glucose by directly emptying intracellular Ca^{2+} stores. PLC also generates diacylglycerol (DAG) that activates protein kinase C (PKC) that augments insulin secretion. Hormones like glucagon and glucagon-like peptide (GLP-1) activate adenylyl cyclase (AC) that produces cyclic AMP. This important second messenger activates protein kinase A (PKA) and the cAMP-sensor protein EPAC2, which collectively stimulate several steps in the insulin release process. By contrast, inhibitors of insulin secretion, e.g. adrenaline, lower insulin secretion by reducing AC activity, leading to decreased cAMP and downstream effects. Not shown here are the additional inhibitory effects of adrenalin on membrane potential and on the exocytotic machinery via phosphatase calcineurin.

actions: first, they activate GTP-protein-regulated inward rectifier K^+ channels that repolarize the membrane potential [94]. The onset of this effect is rapid (within seconds), but lasts only for at most a few minutes. Second, these inhibitors lower cytoplasmic cAMP levels [93], leading to decreased signalling via PKA and EPAC2, which in turn decreases insulin secretion. Third, several studies have presented evidence for the Ca^{2+} -dependent phosphatase calcineurin being an important mediator of direct inhibition of the exocytotic machinery [16, 50, 89]. This fits with the concept that an increased kinase activity leading to an overall increased phosphorylation state of the exocytotic machinery will enhance Ca^{2+} -dependent exocytosis of insulin, whilst activation of phosphatases that have the opposite effect will put a brake on insulin secretion [4] (Fig. 2.6).

2.3 The Role of the Pancreatic β -Cell in Type 2 Diabetes and Future Challenges for β -Cell Research

Type 2 diabetes is easily diagnosed as a chronic elevation in plasma glucose, most easily demonstrated by sampling plasma glucose under fasting conditions or after an oral glucose challenge. The disease is complex, meaning that its clinical characteristics and progression exhibits strong variation between individuals. It is also well known that it is multifactorial, and that diabetes risk increases by environmental factors such as excess caloric intake and low physical activity which lead to obesity. Obesity is associated with increased production of hormones and cytokines from the adipocytes, coined adipokines, such as leptin, adiponectin, TNF- α , interleukin 6 and more. Although much work is still required to detail the exact actions of these adipokines, accumulating evidence suggests that they start off a vicious circle in which increased body fat mass leads to insulin resistance in the main insulin target organs, i.e. skeletal muscle, liver and adipose tissue. As a consequence more insulin has to be released to maintain control over blood glucose.

However, type 2 diabetes also has a strong genetic component and the recently published GWAS for type 2 diabetes have identified a large number of common genetic variations (SNPs) in hundreds of genes that reveal significant association to the disease [28, 98, 103, 128, 129]. These results underscore that type 2 diabetes should be regarded as an umbrella diagnosis with several disease subtypes that may show remarkable variation in terms of pathogenic mechanisms (Fig. 2.7).

Interestingly, the majority of the genes identified in the genetic scans are expected to affect pancreatic islet function. This is in line with the results in the UK Prospective Diabetes Study, which unequivocally demonstrated that a dramatic fall in glucose-evoked insulin secretion precedes elevation of blood glucose and is the event that precipitates type 2 diabetes [115]. As a result today few would neglect the central role of the pancreatic islet in type 2 diabetes. Pancreatic islet failure in type 2 diabetes can emanate from a decrease in the number of insulin-producing β -cells or a deterioration in β -cell function. Studies in pancreatic tissue collected from autopsies have indicated that type 2 diabetes is associated with an average 40–60% reduction

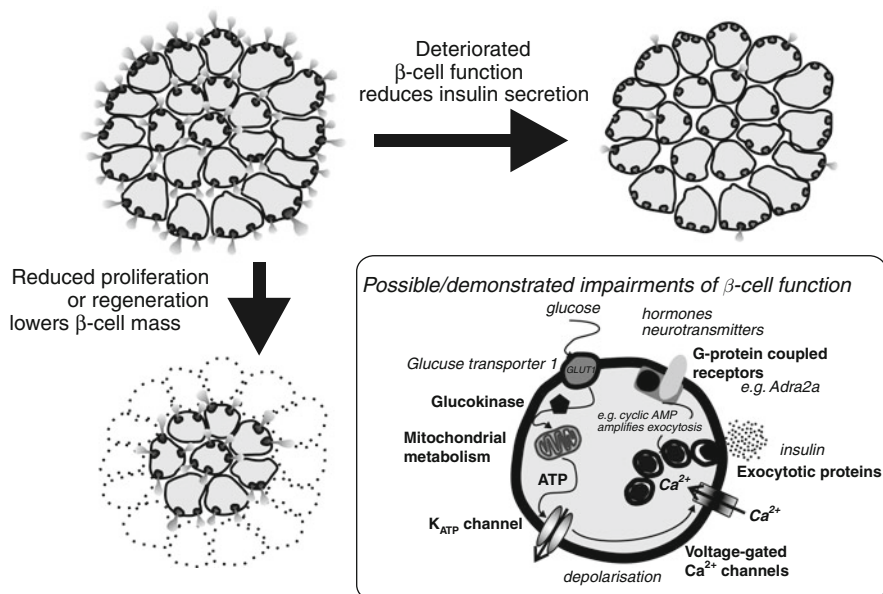


Fig. 2.7 Possible β -cell defects leading to decreased insulin secretion and type 2 diabetes. Deteriorated β -cell function and/or decreased β -cell mass contribute to reduced insulin secretion causally related to type 2 diabetes. Some possible functional defects are shown in the inset, including the recently demonstrated inherited hyperfunction of α -2A adrenoreceptor (Adra2a) signalling.

in β -cell volume, which is due to an increased rate of programmed cell death, or apoptosis, in β -cells [18]. Similar decreases in β -cell mass in type 2 diabetes have been reported in several laboratories, but the interpretation of these results remains a matter of debate [88, 97]. This is because the inter-individual variation is large and the overlap between non-diabetic and diabetic patients is substantial. Furthermore, type 2 diabetes is not a common finding in patients that have undergone partial (30–50%) pancreatectomy [104]. In patients that have removed more than 60% of their pancreas for organ donation, the majority remain normoglycaemic even after 6–18 years [92, 100]. Finally, in longitudinal studies, the decrease in β -cell volume in newly diagnosed patients (1–5 years after diagnosis) is only 26%. However, β -cell volume decreases with duration of the disease and is likely to contribute to secondary failure, i.e. the end stage of type 2 diabetes when oral pharmacological treatment fails to control plasma glucose levels and has to be supplemented with insulin treatment [88]. These findings cast some doubt over the notion that a decrease in β -cell mass/volume is the sole explanation of the deterioration in insulin secretion leading to type 2 diabetes. By contrast, some evidence exists supporting that a decreased β -cell function plays a role in the pathogenesis of type 2 diabetes. Recently, it was demonstrated that a common genetic variation in the gene for the

adrenaline receptor α -2A leads to expression of an increased number such receptors in the islet and, as a consequence, decreased insulin secretion [96]. This finding is valid in both rats and humans and is furthermore associated with an increased risk of type 2 diabetes. Similarly, for TCF7L2, the most important type 2 diabetes gene known to date, a decreased β -cell response to GLP-1 rather than an overall decrease in glucose-induced insulin secretion appears as the main determinant of type 2 diabetes [64].

In summary, these studies indicate that impaired pancreatic β -cell function can never be neglected when discussing the pathogenesis of any case of type 2 diabetes; the pancreatic β -cell can fail in many different ways; and, the ideal treatment may be completely different in patients with different genetic make-up. The main consequence of these lessons learnt is that an important task for β -cell research in the years to come is elucidating the function of the genes that harbour the SNPs associating with disease, as well as to pinpoint how these genetic variations decrease β -cell function and lead to type 2 diabetes. Such knowledge would open up the way for future personalized treatment of the disease, aiming at correcting the cellular reactions that fail in the individual patient.

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Chapter 3

Mitochondria and Metabolic Signals in β -Cells

Pierre Maechler

Abstract Pancreatic β -cells are able to sense glucose and other nutrient secretagogues to regulate insulin exocytosis, thereby maintaining glucose homeostasis. This systems biology of insulin secretion controls translation of metabolic signals into intracellular messengers recognized by the exocytotic machinery. Central to this metabolism-secretion coupling, mitochondria integrate and generate metabolic signals, connecting glucose recognition to insulin exocytosis. In response to a glucose rise, nucleotides and metabolites are generated by mitochondria and participate, together with cytosolic calcium, in the stimulation of insulin release. This chapter describes the mitochondrion-dependent systems of regulated insulin secretion.

Keywords Pancreatic β -cell · Insulin secretion · Diabetes · Mitochondria · Amplifying pathway · Glutamate · Reactive oxygen species

3.1 Introduction

Glucose homeostasis depends on the normal regulation of insulin secretion from the β -cells and the action of insulin on its target tissues. Such equilibrated balance requires tight coupling between glucose metabolism and insulin secretory response. The exocytotic process is tightly controlled by signals generated by nutrient metabolism, as well as by neurotransmitters and circulating hormones. In a systems biology fashion, the β -cell is poised to rapidly adapt the rate of insulin secretion to fluctuations in the blood glucose concentration. This chapter describes the molecular basis of metabolism-secretion coupling. In particular, we will see how mitochondria function both as sensors and generators of metabolic signals.

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3.2 Overview of Metabolism-Secretion Coupling

In the consensus model of glucose-stimulated insulin secretion (Fig. 3.1), glucose equilibrates across the plasma membrane and is phosphorylated by glucokinase, thereby initiating glycolysis [1]. Subsequently, mitochondrial metabolism generates ATP, which promotes the closure of ATP-sensitive K^+ channels (K_{ATP} -channel) and, as a consequence, depolarization of the plasma membrane [2]. This leads to Ca^{2+} influx through voltage-gated Ca^{2+} channels and a rise in cytosolic Ca^{2+} concentrations, which triggers exocytosis of insulin [3].

Additional signals are necessary to sustain the secretion elicited by glucose. They participate in the amplifying pathway [4], formerly referred to as the K_{ATP} -channel-independent stimulation of insulin secretion. Efficient coupling of glucose recognition to insulin secretion is ensured by the mitochondrion, an organelle that integrates and generates metabolic signals. This crucial role goes far beyond the generation of ATP necessary for the elevation of cytosolic Ca^{2+} [5]. The additional coupling factors amplifying the action of Ca^{2+} (Fig. 3.1) will be discussed in this chapter.

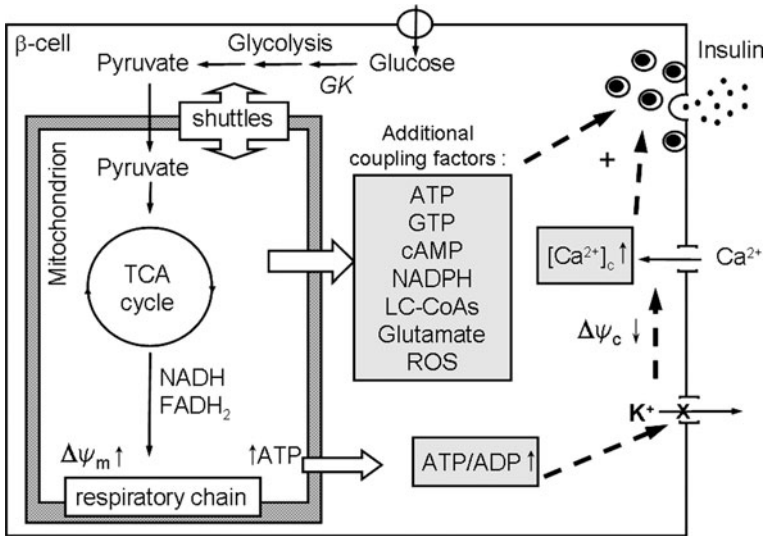


Fig. 3.1 Model for coupling of glucose metabolism to insulin secretion in the β -cell. Glucose equilibrates across the plasma membrane and is phosphorylated by glucokinase (GK). Further, glycolysis produces pyruvate, which preferentially enters the mitochondria and is metabolized by the TCA cycle. The TCA cycle generates reducing equivalents (NADH, $FADH_2$), which are transferred to the electron transport chain, leading to hyperpolarization of the mitochondrial membrane ($\Delta\psi_m \uparrow$) and generation of ATP. ATP is then transferred to the cytosol, raising the ATP/ADP ratio. Subsequently, closure of K_{ATP} -channels depolarizes the cell membrane ($\Delta\psi_c \downarrow$). This opens voltage-dependent Ca^{2+} channels, increasing cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c \uparrow$), which triggers insulin exocytosis. Additive signals participate to the amplifying pathway of metabolism-secretion coupling.

3.3 Mitochondrial NADH Shuttles as Metabolic Sensors

In the cytosolic compartment, glycolysis produces reducing equivalents in the form of NADH. Then, maintenance of glycolytic flux requires reoxidation of NADH to NAD^+ . In most tissues, lactate dehydrogenase ensures NADH oxidation to avoid inhibition of glycolysis secondary to the lack of NAD^+ (Fig. 3.2). In β -cells, which exhibit low lactate dehydrogenase activity [6], high rates of glycolysis are maintained through the activity of mitochondrial NADH shuttles, thereby transferring glycolysis-derived electrons to mitochondria [7]. Therefore, NADH shuttles couple glycolysis to activation of mitochondrial energy metabolism, leading to insulin secretion.

The NADH shuttle system is composed essentially of the glycerol phosphate and the malate/aspartate shuttles [8], with its respective key members mitochondrial glycerol phosphate dehydrogenase and aspartate-glutamate carrier (AGC). Mice lacking mitochondrial glycerol phosphate dehydrogenase exhibit a normal

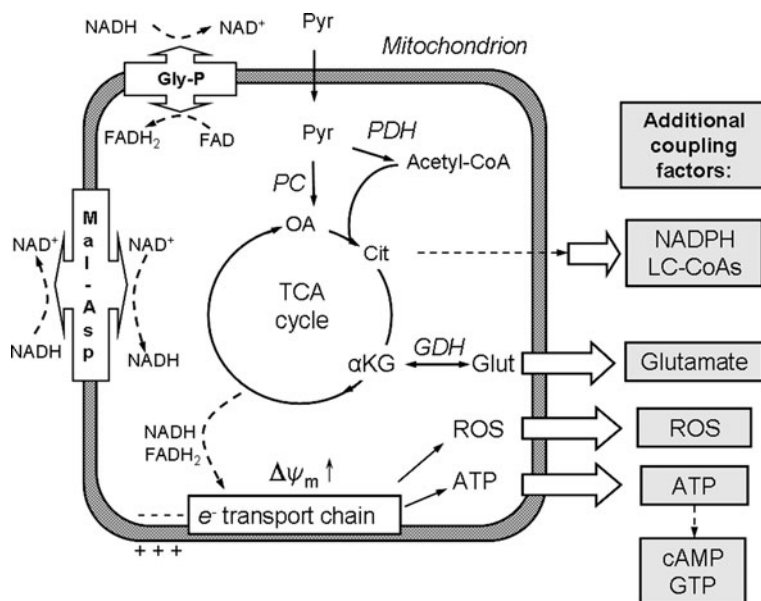


Fig. 3.2 In the mitochondria, pyruvate (Pyr) is a substrate both for pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC), forming respectively acetyl-CoA (Ac-CoA) and oxaloacetate (OA). Condensation Ac-CoA with OA generates citrate (Cit) that is either processed by the TCA cycle or exported out of the mitochondrion as a precursor for long-chain acyl-CoA (LC-CoA) synthesis. Glycerophosphate (Gly-P) and malate/aspartate (Mal-Asp) shuttles as well as the TCA cycle generate reducing equivalents in the form of NADH and FADH_2 , which are transferred to the electron transport chain resulting in hyperpolarization of the mitochondrial membrane ($\Delta\psi_m$) and ATP synthesis. As a by-product of electron transport chain activity, reactive oxygen species (ROS) are generated. Upon glucose stimulation, glutamate (Glut) can be produced from α -ketoglutarate (α KG) by glutamate dehydrogenase (GDH).

phenotype [9], whereas general abrogation of AGC results in severe growth retardation, attributed to the observed impaired central nervous system function [10]. Islets isolated from mitochondrial glycerol phosphate dehydrogenase knockout mice respond normally to glucose regarding metabolic parameters and insulin secretion [9]. Additional inhibition of transaminases with aminooxyacetate, to non-specifically inhibit the malate/aspartate shuttle in these islets, strongly impairs the secretory response to glucose [9]. The respective importance of these shuttles is indicated in islets of mice with abrogation of NADH shuttle activities, pointing to the malate/aspartate shuttle as essential for both mitochondrial metabolism and cytosolic redox state.

Aralar1 (or aspartate-glutamate carrier 1, AGC1) is a Ca^{2+} -sensitive member of the malate/aspartate shuttle [11]. Aralar1/AGC1 and citrin/AGC2 are members of the subfamily of Ca^{2+} -binding mitochondrial carriers and correspond to two isoforms of the mitochondrial aspartate-glutamate carrier. These proteins are activated by Ca^{2+} [12], acting on the external side of the inner mitochondrial membrane [11, 13]. Adenoviral-mediated overexpression of Aralar1/AGC1 increases glucose-induced mitochondrial activation and secretory response, both in insulinoma INS-1E cells and rat islets [14]. This is accompanied by enhanced glucose oxidation and reduced lactate production. Recently, we conducted the mirror experiment by downregulating Aralar1/AGC1 in the same cell models [15]. In INS-1E cells, Aralar1/AGC1 knockdown reduced glucose oxidation and the secretory response, although rat islets were not sensitive to such a manoeuvre [15]. Taken as a whole, aspartate-glutamate carrier capacity appears to set a limit for NADH shuttle function and mitochondrial metabolism, exhibiting cell-specific dependence. The importance of the NADH shuttle system also illustrates the tight coupling between glucose catabolism and insulin secretion.

3.4 Getting In and Out of the Tricarboxylic Acid Cycle

In pancreatic β -cells, high NADH shuttle activity favours transfer of the glycolysis product pyruvate into mitochondria. Pyruvate import into the mitochondrial matrix is associated with a futile cycle that transiently depolarizes the mitochondrial membrane [16]. After its entry into mitochondria, pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase or to oxaloacetate by pyruvate carboxylase (Fig. 3.2). The pyruvate carboxylase pathway ensures the provision of carbon skeleton (i.e. anaplerosis) to the tricarboxylic acid (TCA) cycle, a key pathway in β -cells [17–20]. Noteworthy, inhibition of the pyruvate carboxylase reduces glucose-stimulated insulin secretion in rat islets [21]. The very high anaplerotic activity suggests important loss of TCA cycle intermediates (i.e. cataplerosis), compensated for by pyruvate carboxylation to synthesize *de novo* oxaloacetate. In the control of glucose-stimulated insulin secretion, TCA cycle intermediates might serve as substrates leading to the formation of mitochondria-derived coupling factors [5].

Importance of TCA cycle activation for β -cell function is illustrated by stimulation with substrates bypassing glycolysis. This is the case for the TCA

cycle intermediate succinate, or its cell permeant methyl derivatives, that has been shown to efficiently promote insulin secretion in pancreatic islets [22, 23]. Succinate induces hyperpolarization of the mitochondrial membrane, resulting in elevation of mitochondrial Ca^{2+} and ATP generation, while its catabolism is Ca^{2+} dependent [22].

The mitochondrion in general, and the TCA cycle in particular, is the key metabolic crossroad enabling fuel oxidation as well as provision of building blocks, or cataplerosis, for lipids and proteins [24]. In β -cells, approximately 50% of pyruvate is oxidized to acetyl-CoA by pyruvate dehydrogenase [18]. Pyruvate dehydrogenase is an important site of regulation as, among other effectors, the enzyme is activated by elevation of mitochondrial Ca^{2+} [25, 26] and, conversely, its activity is reduced upon exposures to either excess fatty acids [27] or chronic high glucose [28]. Oxaloacetate condenses with acetyl-CoA forming citrate, which undergoes stepwise oxidation and decarboxylation yielding α -ketoglutarate. The TCA cycle is completed via succinate, fumarate, and malate, in turn producing oxaloacetate (Fig. 3.2). The fate of α -ketoglutarate is influenced by the redox state of mitochondria. Low NADH to NAD^+ ratio would favour further oxidative decarboxylation to succinyl-CoA as NAD^+ is required as cofactor for this pathway. Conversely, high NADH to NAD^+ ratio would promote NADH-dependent reductive transamination forming glutamate, a spin-off product of the TCA cycle [24]. The latter situation, i.e. high NADH generated at the expense of NAD^+ , is a physiological consequence of glucose stimulation in β -cells [29, 30].

Although the TCA cycle also oxidizes fatty acids and amino acids, carbohydrates are the most important fuel under physiological conditions for the β -cell. Upon glucose exposure, mitochondrial NADH elevations reach a plateau after approximately 2 min [31]. In order to maintain pyruvate input into the TCA cycle, this new redox steady state requires continuous reoxidation of mitochondrial NADH to NAD^+ , primarily by complex I of the electron transport chain. However, as complex I activity is limited by the inherent thermodynamic constraints of proton gradient formation [32], excess NADH contributed by this high TCA cycle activity must be reoxidized by other dehydrogenases, i.e. through cataplerotic reactions. Indeed, significant cataplerotic activity in β -cells was suggested by the quantitative importance of anaplerotic pathways employing pyruvate carboxylase [17, 18], as confirmed by use of NMR spectroscopy [19, 20, 33].

3.5 Mitochondrial Control of the Glutamate Dehydrogenase

The enzyme glutamate dehydrogenase (GDH) is a key enzyme in the control of the secretory response (Fig. 3.2). GDH is a homohexamer located in the mitochondrial matrix and catalyses the reversible reaction α -ketoglutarate + NH_3 + NADH $\leftrightarrow$ glutamate + NAD^+ ; inhibited by GTP and activated by ADP [34, 35]. In the β -cell, allosteric activation of GDH has received most of the attention over the past three decades [36]. Numerous studies have used the GDH allosteric activator L-leucine

or its non-metabolized analogue β -2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) to address the role of GDH in the control of insulin secretion [36–39]. Alternatively, GDH activity can be increased by means of overexpression, an approach that we combined with allosteric activation of the enzyme [40]. To date, the specific role of GDH in β -cell function remains unclear. GDH participates in the glucose-induced amplifying pathway through generation of glutamate [41–43]. The enzyme is also an amino acid sensor triggering insulin release upon glutamine stimulation under conditions of GDH allosteric activation [37, 39, 44].

More recently, the importance of GDH has been further highlighted by studies showing that SIRT4, a mitochondrial ADP-ribosyltransferase, downregulates GDH activity and thereby modulates insulin secretion [45, 46]. Clinical data and associated genetic studies also revealed GDH as a key enzyme for the control of insulin secretion. Indeed, mutations rendering GDH more active are responsible for a hyperinsulinism syndrome [47]. Mutations producing a less active, or even non-active, GDH enzyme have not been reported, leaving the question open if such mutations would be either lethal or asymptomatic. We recently generated and characterized transgenic mice (named β Glud1^{-/-}) with a β -cell-specific deletion of GDH [48]. Data show that loss of GDH in β -cells is associated with a ~40% reduction in glucose-stimulated insulin secretion and that the GDH pathway lacks redundant mechanisms. In β Glud1^{-/-} mice, the reduced secretory capacity resulted in lower plasma insulin levels in response to both feeding and glucose load, while body weight gain was preserved [48]. This demonstrates that GDH is essential for the full development of the secretory response in β -cells, operating in the upper range of physiological glucose concentrations.

3.6 Mitochondrial Activation

TCA cycle activation induces transfer of electrons to the respiratory chain resulting in hyperpolarization of the mitochondrial membrane and generation of ATP (Fig. 3.2). The electrons are transferred by the pyridine nucleotide NADH and the flavin adenine nucleotide FADH₂. In the mitochondrial matrix, NADH is formed by several dehydrogenases, some of which are activated by Ca²⁺ [25], while FADH₂ is generated in the succinate dehydrogenase reaction.

Electron transport chain activity promotes proton export from the mitochondrial matrix across the inner membrane, establishing a strong mitochondrial membrane potential, which is negative on the inside. The respiratory chain comprises five complexes, the subunits of which are encoded by both the nuclear and mitochondrial genomes [49]. Complex I is the only acceptor of electrons from NADH in the inner mitochondrial membrane and its blockade abolishes glucose-induced insulin secretion [32]. Complex II (succinate dehydrogenase) transfers electrons to coenzyme Q from FADH₂, the latter being generated both by the oxidative activity of the TCA cycle and the glycerol phosphate shuttle. Complex V (ATP synthase) promotes ATP formation from ADP and inorganic phosphate. The synthesized ATP is translocated to the cytosol in exchange for ADP by the adenine nucleotide translocator (ANT).

Thus, the actions of the separate complexes of the electron transport chain and the adenine nucleotide translocator couple respiration to ATP supply.

Mitochondrial activity can be modulated according to the nature of the nutrients, although glucose is the chief secretagogue as compared to amino acid catabolism [50] and fatty acid β -oxidation [51]. Additional factors regulating ATP generation include mitochondrial Ca^{2+} levels [25, 52], mitochondrial protein tyrosine phosphatase [53], mitochondrial GTP [54], and matrix alkalization [55].

Mitochondrial activation also involves changes in organelle morphology and contacts. Mitochondria form dynamic networks, continuously modified by fission and fusion events under the control of specific mitochondrial membrane anchor proteins [56]. Mitochondrial fission/fusion state was recently investigated in insulin-secreting cells. Altering fission by downregulation of fission-promoting Fis1 protein impairs respiratory function and glucose-stimulated insulin secretion [57]. The reverse experiment, consisting in overexpression of Fis1 causing mitochondrial fragmentation, results in a similar phenotype, i.e. reduced energy metabolism and secretory defects [58]. Fragmented pattern obtained by dominant-negative expression of fusion-promoting Mfn1 protein does not affect metabolism-secretion coupling [58]. Therefore, mitochondrial fragmentation per se seems not to alter insulin-secreting cells, at least not in vitro.

3.7 The Amplifying Pathway of the Secretory Response

The Ca^{2+} signal in the cytosol is necessary but not sufficient for the full development of sustained insulin secretion. Nutrient secretagogues, in particular glucose, evoke a long-lasting second phase of insulin secretion. In contrast to the transient secretion induced by Ca^{2+} -raising agents, the sustained insulin release depends on the generation of metabolic factors (Fig. 3.1). The elevation of cytosolic Ca^{2+} is a prerequisite also for this phase of secretion, as evidenced among others by the inhibitory action of voltage-sensitive Ca^{2+} channel blockers. Glucose evokes K_{ATP} -channel-independent stimulation of insulin secretion, or the amplifying pathway [4], which is unmasked by glucose stimulation when cytosolic Ca^{2+} is clamped at permissive levels [59–61]. This suggests the existence of metabolic coupling factors generated by glucose.

3.8 Mitochondria-Derived Nucleotides as Coupling Factors

ATP is the primary metabolic factor implicated in K_{ATP} -channel regulation [62], secretory granule movement [63, 64], and the process of insulin exocytosis [65, 66].

Among other putative nucleotide messengers, NADH and NADPH are generated by glucose metabolism [67]. Single β -cell measurements of NAD(P)H fluorescence have demonstrated that the rise in pyridine nucleotides precedes the rise in cytosolic Ca^{2+} concentrations [30] and that the elevation in the cytosol precedes the one in

mitochondria [29]. Cytosolic NADPH is generated by glucose metabolism via the pentose phosphate shunt [68], although mitochondrial shuttles appear to be the main contributors in β -cells [69]. The pyruvate/citrate shuttle has received some attention over the last years and has been postulated as the key cycle responsible for elevation of cytosolic NADPH [69]. As a consequence of mitochondrial activation, cytosolic NADPH is generated by NADP⁺-dependent malic enzyme and suppression of its activity was shown to inhibit glucose-stimulated insulin secretion in insulinoma cells [70, 71]. However, such effects have not been reproduced in primary cells in the form of rodent islets [72], leaving the question open concerning its regulatory role.

Regarding the action of NADPH, it was proposed as a coupling factor in glucose-stimulated insulin secretion based on experiments using toadfish islets [73]. A direct effect of NADPH was reported on the release of insulin from isolated secretory granules [74], NADPH being possibly bound or taken up by granules [75]. More recently, the putative role of NADPH, as a signalling molecule in β -cells, has been substantiated by experiments showing direct stimulation of insulin exocytosis upon intracellular addition of NADPH [76].

Glucose also promotes the elevation of GTP [77], which could trigger insulin exocytosis via GTPases [65, 78]. In the cytosol, GTP is mainly formed through the action of nucleoside diphosphate kinase from GDP and ATP. In contrast to ATP, GTP is capable of inducing insulin exocytosis in a Ca²⁺-independent manner [65]. An action of mitochondrial GTP as positive regulator of the TCA cycle has been mentioned above [54].

The universal second messenger cAMP, generated at the plasma membrane from ATP, potentiates glucose-stimulated insulin secretion [79]. Many neurotransmitters and hormones, including glucagon as well as the intestinal hormones glucagon-like peptide 1 (GLP-1) and gastric insulinotropic polypeptide (GIP), increase cAMP levels in the β -cell by activating adenyl cyclase [80]. In human β -cells, activation of glucagon receptors synergistically amplifies the secretory response to glucose [81]. Glucose itself promotes cAMP elevation [82], and oscillations in cellular cAMP concentrations are related to the magnitude of pulsatile insulin secretion [83]. Moreover, GLP-1 might preserve β -cell mass, both by induction of cell proliferation and by inhibition of apoptosis [84]. According to all these actions, GLP-1 and biologically active related molecules are of interest for the treatment of diabetes [85].

3.9 Fatty Acid Pathways and the Secretory Response

The metabolic profile of mitochondria is modulated by the relative contribution of glucose and lipid products for oxidative catabolism. Carnitine palmitoyltransferase I, which is expressed in the pancreas as the liver isoform (LCPTI), catalyses the rate-limiting step in the transport of fatty acids into the mitochondria for their oxidation. In glucose-stimulated β -cells, citrate exported from the

mitochondria (Fig. 3.2) to the cytosol reacts with coenzyme A (CoA) to form cytosolic acetyl-CoA necessary for malonyl-CoA synthesis. Then, malonyl-CoA derived from glucose metabolism regulates fatty acid oxidation by inhibiting LCPTI. The malonyl-CoA/long-chain acyl-CoA hypothesis of glucose-stimulated insulin release postulates that malonyl-CoA derived from glucose metabolism inhibits fatty acid oxidation, thereby increasing the availability of long-chain acyl-CoA for lipid signals implicated in exocytosis [17]. In the cytosol, this process promotes the accumulation of long-chain acyl-CoAs such as palmitoyl-CoA [86, 87], which enhances Ca^{2+} -evoked insulin exocytosis [88].

In agreement with the malonyl-CoA/long-chain acyl-CoA model, overexpression of native LCPTI in clonal INS-1E β -cells was shown to increase β -oxidation of fatty acids and to decrease insulin secretion at high glucose [51], although glucose-derived malonyl-CoA was still able to inhibit LCPTI in these conditions. When the malonyl-CoA/CPTI interaction is altered in cells expressing a malonyl-CoA-insensitive CPTI, glucose-induced insulin release is impaired [89].

The malonyl-CoA/long-chain acyl-CoA model has been challenged over the last years, essentially by modulating cellular levels of malonyl-CoA, either up or down. Either approach resulted in contradictory results, according to the respective laboratories performing such experiments. First, malonyl-CoA decarboxylase was overexpressed to reduce malonyl-CoA levels in the cytosol. In disagreement with the malonyl-CoA/long-chain acyl-CoA model, abrogation of malonyl-CoA accumulation during glucose stimulation does not attenuate the secretory response [90]. However, overexpression of malonyl-CoA decarboxylase in the cytosol in the presence of exogenous free fatty acids, but not in their absence, reduces glucose-stimulated insulin release [91]. The second approach was to silence ATP-citrate lyase, the enzyme that forms cytosolic acetyl-CoA leading to malonyl-CoA synthesis. Again, one study observed that such a manoeuvre reduces glucose-stimulated insulin secretion [70], whereas another group concluded that metabolic flux through malonyl-CoA is not required for the secretory response to glucose [71].

The role of long-chain acyl-CoA derivatives remains a matter of debate, although several studies indicate that malonyl-CoA could act as a coupling factor regulating the partitioning of fatty acids into effector molecules in the insulin secretory pathway [92]. Fatty acids, mobilized from intracellular triglyceride stores, might also play a permissive role in the secretory response [93, 94]. Moreover, fatty acids stimulate the G-protein-coupled receptor GPR40/FFAR1 that is highly expressed in β -cells [95]. Activation of GPR40 receptor results in enhancement of glucose-induced elevation of cytosolic Ca^{2+} and consequently insulin secretion [96].

3.10 Mitochondria-Derived Metabolites as Coupling Factors

Acetyl-CoA carboxylase catalyses the formation of malonyl-CoA, a precursor in the biosynthesis of long-chain fatty acids. Interestingly, glutamate-sensitive protein phosphatase 2A-like protein activates acetyl-CoA carboxylase in β -cells [97]. This

observation might link two metabolites proposed to participate in the control of insulin secretion. Indeed, the amino acid glutamate is another metabolic factor proposed to participate in the amplifying pathway [41, 42, 98]. Glutamate can be produced from the TCA cycle intermediate α -ketoglutarate or by transamination reactions [35, 50, 99]. During glucose stimulation total cellular glutamate levels have been shown to increase in human, mouse, and rat islets as well as in clonal β -cells [19, 40, 41, 43, 100–102], whereas one study reported no change [103].

The finding that mitochondrial activation in permeabilized β -cells directly stimulates insulin exocytosis [5] initiated investigations that identified glutamate as a putative intracellular messenger [41, 42]. In situ pancreatic perfusion, increased provision of glutamate using a cell permeant precursor results in augmentation of the sustained phase of insulin release [104]. The glutamate hypothesis was challenged by overexpression of glutamate decarboxylase (GAD) in β -cells to reduce cytosolic glutamate levels [100]. In control cells, stimulatory glucose concentrations increased glutamate concentrations, whereas the glutamate response was significantly reduced in GAD overexpressing cells. GAD overexpression also blunted insulin secretion induced by high glucose, showing direct correlation between glutamate changes and the secretory response [100]. In contrast, it was reported by others that glutamate changes may be dissociated from the amplification of insulin secretion elicited by glucose [101]. Recently, we abrogated GDH, the enzyme responsible for glutamate formation, specifically in the β -cells of transgenic mice. This resulted in a 40% reduction of glucose-stimulated insulin secretion [48]. Export of glutamate out of the mitochondria is mediated by a newly identified protein, namely the glutamate carrier GC1 located in the inner mitochondrial membrane [105]. Silencing of GC1 in β -cells inhibits insulin exocytosis evoked by glucose stimulation, an effect rescued by the provision of exogenous glutamate to the cell [105].

The use of selective inhibitors led to a model where glutamate, downstream of mitochondria, would be taken up by secretory granules, thereby promoting Ca^{2+} -dependent exocytosis [41, 42]. Such a model was strengthened by demonstration that clonal β -cells express two vesicular glutamate transporters (VGLUT1 and VGLUT2) and that glutamate transport characteristics are similar to neuronal transporters [106]. The mechanism of action inside the granule could possibly be explained by glutamate-induced pH changes, as observed in secretory vesicles from pancreatic β -cells [107]. An alternative mechanism of action at the secretory vesicle level implicates glutamate receptors. Indeed, clonal β -cells have been shown to express the metabotropic glutamate receptor mGlu5 in insulin-containing granules, thereby mediating insulin secretion [108].

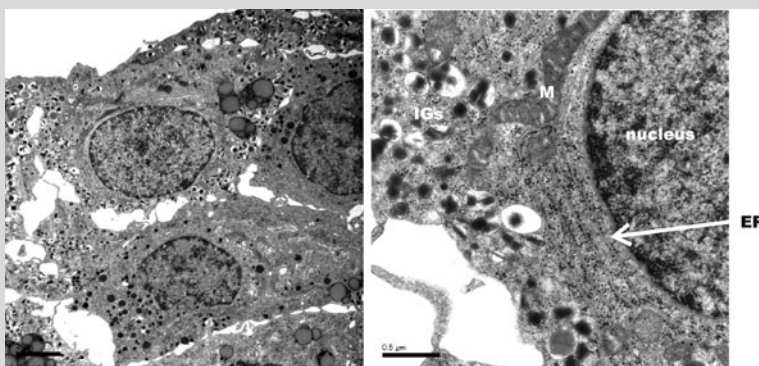
Another action of glutamate has been proposed. In insulin-secreting cells, rapidly reversible protein phosphorylation/dephosphorylation cycles have been shown to play a role in the rate of insulin exocytosis [109]. It has also been reported that glutamate, generated upon glucose stimulation, might sustain glucose-induced insulin secretion through inhibition of protein phosphatase enzymatic activities [102]. Finally, an alternative or additive mechanism of action would be activation of acetyl-CoA carboxylase [97] as mentioned above.

Panorama of β -Cell Organelles

The pancreatic β -cell is the main glucose sensor and insulin factory of the body. All standard cellular organelles are expressed in the β -cell, but its specific tasks have consequences for the relative abundance of the different organelles. The high rates of insulin synthesis are visible in high-resolution electron micrographs as a well-developed endoplasmatic reticulum (ER) and Golgi apparatus. Under conditions when the demand for insulin exceeds the capacity of the β -cell, signs of ER stress with luminal swelling may appear.

The glucose sensor input function is reflected as a high density of mitochondria, which generate ATP from metabolites of the internalized glucose molecules.

Regulated exocytosis of stored insulin granules represents the output signal of the β -cell. The mature insulin granule appears with a dark dense core and a halo region, whereas immature granules have a more opaque appearance.



Electron micrograph of a human pancreatic islet. In the left image, one β -cell can be seen (top, left), as well as two glucagon-producing α -cells. Scale bar 2 μ m.

In the right image, part of the β -cell is shown at higher magnification. Clearly visible is the endoplasmatic reticulum (ER), a mitochondrion (M), and dense-core insulin granules (IGs). Scale bar 0.5 μ m.

Images made and generously shared by Dr. Lena Eliasson, Lund University.

Added by the editors

Several mechanisms of action have been proposed for glutamate as a metabolic factor playing a role in the control of insulin secretion. However, we lack a consensus model and further studies should dissect these complex pathways that might be either additive or cooperative.

Among mitochondrial metabolites, citrate export out of the mitochondria has been described as a signal of fuel abundance. Such a cataplerotic pathway might participate in β -cell metabolism-secretion coupling [69]. In the cytosol, metabolism

of citrate contributes to the formation of NADPH and malonyl-CoA, both proposed as metabolic coupling factors as discussed above.

3.11 Reactive Oxygen Species Participate to β -Cell Function

Reactive oxygen species (ROS) include superoxide ($O_2^- \bullet$), hydroxyl radical ($OH \bullet$), and hydrogen peroxide (H_2O_2). Superoxide can be converted to less reactive H_2O_2 by superoxide dismutase (SOD) and then to oxygen and water by catalase (CAT), glutathione peroxidase (GPx), and peroxiredoxin, which constitute antioxidant defences.

Mitochondrial electron transport chain is the major site of ROS production within the cell. Electrons from sugar, fatty acid, and amino acid catabolism accumulate in the electron carriers NADH and $FADH_2$, and are subsequently transferred through the electron transport chain to oxygen, promoting ATP synthesis. ROS formation is coupled to this electron transportation as a by-product of normal mitochondrial respiration through the one-electron reduction of molecular oxygen [110, 111]. The main sub-mitochondrial localization of ROS formation is the inner mitochondrial membrane, i.e. NADH dehydrogenase at complex I and the interface between ubiquinone and complex III [112]. Increased mitochondrial free radical production has been regarded as a result of diminished electron transport occurring when ATP production saturates the system or under certain stress conditions impairing specific respiratory chain complexes [113, 114].

ROS may exert different actions according to cellular concentrations being either below or above a specific threshold, i.e. signalling or toxic effects respectively. Robust oxidative stress, caused either by direct exposure to oxidants or secondary to glucolipotoxicity, has been shown to impair β -cell function [115–117]. Specifically, ROS attacks in insulin-secreting cells result in mitochondrial inactivation, thereby interrupting transduction of signals normally coupling glucose metabolism to insulin secretion [115]. Even one single acute oxidative stress can induce β -cell dysfunction lasting over days, explained by persistent damages in mitochondrial components accompanied by subsequent generation of endogenous ROS of mitochondrial origin [118].

However, metabolism of physiological nutrient increases ROS without causing deleterious effects on cell function. Recently, the concept emerged that ROS might participate in cell signalling [119]. In insulin-secreting cells, it has been reported that ROS, and probably H_2O_2 in particular, is one of the metabolic coupling factor in glucose-induced insulin secretion [120]. Therefore, ROS fluctuations may also contribute to physiological control of β -cell functions.

3.12 Conclusion

Mitochondria are key organelles that generate the largest part of cellular ATP and represent the central crossroad of metabolic pathways. Metabolic profiling of β -cell

function identified mitochondria as sensors and generators of metabolic signals controlling insulin secretion (see also Box “Panorama of β -Cell Organelles”). Recent molecular tools available for cell biology studies shed light on new mechanisms regarding the coupling of glucose recognition to insulin exocytosis. Delineation of metabolic signals required for β -cell function will be instrumental in drawing the map of the systems biology of insulin secretion.

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Chapter 4

β -Cell Ontogenesis and the Insulin Production Apparatus

R. Scott Heller and Ole D. Madsen

Abstract The pancreatic insulin-producing β -cell is a highly specialized cell that develops into the main endocrine cell types in the islets of Langerhans of the pancreas from the primitive gut endoderm. A large number of specific transcription factors have been demonstrated to be crucial to the development and function of this highly specialized cell. Recently, genome-wide association scans as well as study of maturity onset diabetes of the young genes has demonstrated that most of these genes are expressed in the pancreatic β -cell and are involved in not only transcriptional functions but also the insulin secretory apparatus. This chapter provides a short overview of these subjects.

Keywords System biology · Genome wide association · Transcription factors · Development · Phylogenetic · Diabetes · Maturity onset diabetes of the young · Pancreatic progenitors

4.1 Early Pancreatic Organogenesis

Pancreatic organogenesis is a largely conserved process throughout vertebrate development. Phylogenetic studies of pancreas [19] suggest that the insulin-producing β -cell founded the pancreatic organ (together with few somatostatin-producing cells and cytokeratin-immunoreactive cells (Christensen, Madsen, and Heller, unpublished data) and the alpha cells, acinar cells, and the PP cells entered at later stages [10, 29] (Fig. 4.1). Shortly after gastrulation and formation of the endoderm both ventral and dorsal regions initiate pancreas formation (pancreatic anlage) ([48] – for review). The ventral pancreatic bud becomes the head of the pancreas while the dorsal bud becomes the tail. In certain fish species, such as salmonid fish, the dorsal part primarily forms a giant islet structure, the Brockmann body

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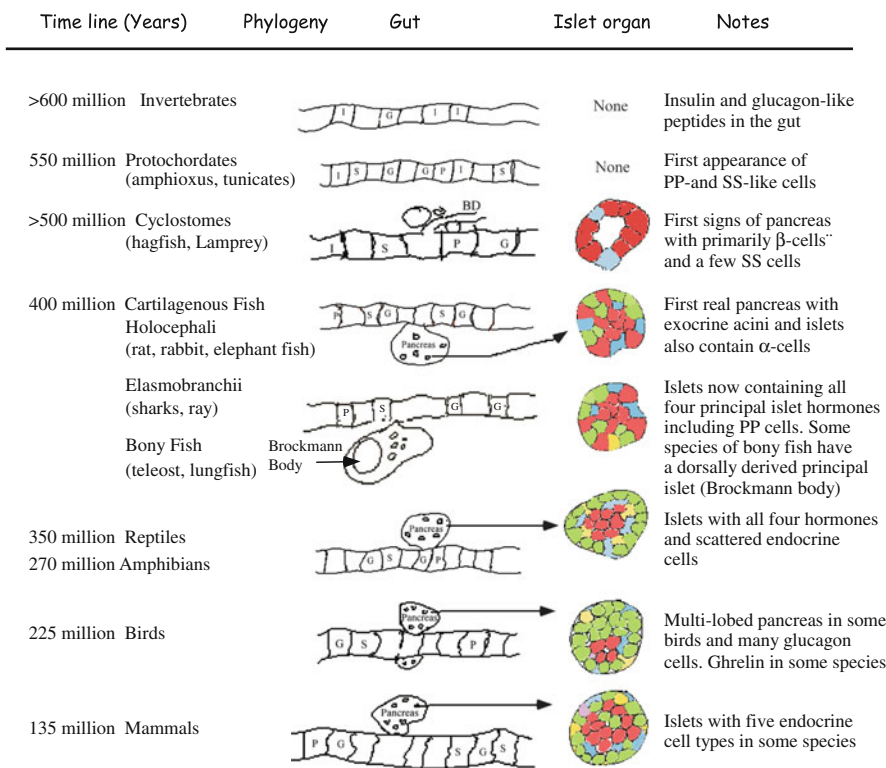


Fig. 4.1 Evolution of the islet organ from invertebrates to mammals. Considerable species variation occurs in all classes but the scheme is meant to be semi-representative. Family member cell types that still remain in the gut are represented by single letters: I = insulin, G = glucagon peptides, SS = somatostatin peptides, and P = PP family peptides. The cyclostomes are the first species where islet like clusters have migrated out of the gut tube into a separate cluster (islet) surrounding the common bile duct. It is with the cartilaginous and bony fish that the first real pancreas is formed with islets containing three and sometimes four hormones. These islets can lie within large islets (Brockmann bodies) or multiple islets within an exocrine pancreas. Reptiles and amphibians are the first species with islets containing all four of the major hormones. Some species of Aves have multi-lobed pancreata and the islets tend to contain a lot of glucagon cells and this is the first appearance of ghrelin cells in some species. Mammals have a diverse range of structures but are generally round and contain four or five islet hormones. Insulin (red), glucagon (green), somatostatin (blue), pancreatic polypeptide (yellow), ghrelin (purple). BD = bile duct. Modified from Falkmer et al. [10] and Heller [19].

(which becomes embedded in ventrally derived pancreatic exocrine parenchyma). Ventral and dorsal origins of pancreas are likely governed by distinct mechanisms of specification [42] and different cues may appear to control the subsequent development of pancreatic tissue that otherwise appears almost identical in the head and tail regions – maybe with the exceptions of the islet composition where more PP-rich islet appears in the head region in contrast to more alpha-cell-rich islet in the tail region. Pdx-1 is first detected in the earliest pancreatic anlage (i.e. at e8.5–e9

in dorsal and ventral buds of the mouse pancreas) [22]. Its expression subsequently expands to comprise duodenal and antral stomach tissues. Pdx-1 deficiency causes pancreas agenesis (in zebrafish, mouse, and man) [21, 33, 44]. Subsequent to the onset of Pdx-1 expression, two additional transcription factors become activated including Nkx6.1 (restricted to the β -cells in the adult pancreas) and Ptf1a (restricted to the acinar cells of the adult pancreas). At this stage most of the cells in the buds co-express all three factors [18] – and such cells are considered to represent multipotent pancreatic progenitors (see Fig. 4.1) that subsequently can give rise to all mature pancreatic cell types [4, 49]. A new role of sox17 was recently reported by the Wells research group. Importantly, they are able to demonstrate that Sox17 is critical for the proper segregation of Pdx-1 progenitors to the ventral pancreas and not the liver or biliary tract [42].

4.2 Expansion of Progenitors

While the early buds still form in the absence of Pdx-1 [21, 33], they fail to proliferate and the Pdx-1-null phenotype is reminiscent of that of FGF10-nulls [2], suggesting that FGF10 is responsible for the proliferation of the earliest pancreatic progenitors. Profound expansion of the triple-positive cells leads to the formation of a multilayered “squamous” epithelium where luminal cavities and polarization of epithelial cells commence [23]. Concomitantly, the process of branching morphogenesis characterizes the following period of pancreas development where true epithelial layer forms and expands by branching. During branching morphogenesis, there is a stringent segregation of the expression domains for Nkx6.1 and Ptf1a such that Nkx6.1 remains within the trunks of the branches while Ptf1a dominates in the tips [18]. The endocrine cells will subsequently arise in the trunk-domain (dependent on Ngn3 activation) [14, 17] while the tip of the branches will form the acini [46].

4.3 Early Differentiation

Pancreatic endocrine differentiation in rodents is described to occur in two phases – the primary and the secondary transition [22, 34]. During the primary transition, mature (based on EM morphology) alpha cells are formed in readily detectable numbers. These early glucagon cells express the prohormone-converting enzymes PC1/3 in addition to PC2 [26, 46] – in contrast to the adult alpha cell (only expressing PC2, required for glucagon processing [12]. As a consequence the early glucagon cells produce glucagon as well as GLP-1 and GLP-2 [25]. It is plausible that early glucagon cells later down-regulate expression of PC1/3 and contribute to the adult-type alpha cells in the mature islets. Also early insulin gene activity is measurable as mRNA and immunoreactive insulin during the primary transition. Early reports on the existence of early multi-hormonal endocrine cells co-expressing glucagon

and low levels of insulin suggested the existence of a multipotent endocrine multi-hormonal progenitor. Elegant lineage tracing studies by Herrera demonstrated that insulin and glucagon cells arise from distinct lineages that diverge prior to the onset of hormone gene expression [20]. The nature of double hormone-positive cells during development remains elusive. β -cells primarily arise during the secondary transition (e13–15) as described below and occur in the trunk region characterized by Nkx6.1 and Pdx-1 expression [22].

4.4 The Choice to Become a β -Cell

Once the endocrine precursor cells (Ngn3⁺) progress beyond a very early phase, a serious choice is forced upon them. What endocrine cell type will I become: insulin, glucagon, somatostatin, pancreatic polypeptide or ghrelin? Recent evidence suggests that this is already predetermined [9], while others support a model where a balance between Pax4 and Arx is the most critical decision on what will turn the Ngn3⁺ cell into either β - or α -cells [24].

Very eloquent *in vivo* clonal analysis experiments of Ngn3-expressing cells demonstrated that every Ngn3 cell becomes one endocrine cell type with a restricted and specific differentiation potential that is determined at a very early stage [9]. A future perspective is to better understand if there is a certain gene expression profile that marks cells for a specific fate prior to Ngn3 activation or if it is the ability of that single cell to respond to specific extracellular cues that drives this progression to becoming a β -cell. Understanding these things will be crucial to directed differentiation of stem cells to pancreatic β -cells.

The importance of the Arx and Pax4 transcription factors in cell fate decisions have been well elucidated in the past 13 years since the publication of the Pax4 knockout mouse [41]. Arx promotes the glucagon/pancreatic polypeptide cell fates, while Pax4 induces insulin and somatostatin cell fates [5–8, 24]. In a series of very well-conducted experiments, Collombat and colleagues have been able to demonstrate that Pax4 is not absolutely required to specify the somatostatin and insulin cell fates but acts by inhibiting the glucagon cell fate, which studies have shown to be the first hormone cell type (or default) that is created in the genesis of endocrine cells [22]. Additionally, Pax4 is able to direct endocrine cells into the β -cells, even mature glucagon cells [8, 27].

4.5 Young β -Cells

Once high-level Nkx6.1 and Pdx-1 expression is observed, this is a strong sign that an endocrine cell is committed to become a β -cell. A number of specific transcription factors (IA-1, Nkx2.2, Pax6) are important for the β -cell identity and mutations in these create endocrine cells without the expression of insulin. IA-1 is a direct target of Ngn3 and has been shown to be necessary but not sufficient for endocrine

cells. Mice lacking IA-1 still have endocrine cells but most are lacking hormones, while overexpression studies alone do not induce endocrine cell formation [13, 31].

Nkx6.1 is expressed broadly in the developmental pancreatic trunk epithelium and specifically plays a role in the mature β -cells, as knockout of the gene has a phenotype where mice are devoid of late but not early created β -cells [38]. Pax4 is a very important factor in promoting the β -cell fate. While Pax4 is not directly required to specify the β -cell but rather blocks the α -cell fate, it has powerful effects in endocrine precursors to drive β -cell differentiation [8, 24]. Nkx2.2 also specifically affects the β -cell fate and Pax4-Nkx2.2 double knockout mice have the same β -cell phenotype, which suggests that Nkx2.2 is acting upstream of Pax4 [35, 45].

The earliest β -cells found in late embryonic life and early post-natal development are characterized by the ability to proliferate, a feature that becomes severely decreased just after weaning in animals. Thereafter, the β -cell mass is maintained by a very slow level of replication [15]. In the recent *in vivo* clonal analysis paper, following Ngn3 cell fates, it was also recognized that β -cells proliferate at a very slow rate and that the number of islets remains constant during 2–10 months of life [9]. This has also been demonstrated in Ob/Ob mice [3].

4.6 Mature β -Cells

Once the β -cell has matured, it is an extremely metabolically active cell which has to precisely function to deliver insulin in direct response to the blood glucose levels to maintain glucose homeostasis within a very precise range. The insulin apparatus has been honed after many millions of years of evolution. Many transcription factors have been shown to be very important in the regulation of glucose-stimulated insulin secretion and these include MafA, FoxO1, Pdx-1, and others (see recent review by Shao et al. [39]). Furthermore, the demonstration that so many single mutations in β -cell genes can directly disrupt the function only highlights the importance of the high demands and precise regulation that is required in the β -cell.

The ability of the β -cell to adapt to physiological and pathophysiological conditions such as pregnancy and obesity increases the demand for insulin. The β -cell under normal circumstances responds with increased biosynthesis of insulin and increased replication of β -cell numbers. Peripheral insulin resistance (obesity) triggers a compensatory up-regulation of β -cell mass [30, 36]. In mice this process appears to be driven via the insulin receptor on the β -cell [1] and requiring IRS2 to mediate the mitotic signal [47]. Insulin itself is thus an obvious candidate as a positive feedback growth signal for the β -cells, which makes sense as long as glucose level is above near-normal range.

Maturity onset diabetes of the young (MODY) is described as having the following characteristics: a primary defect in insulin secretion and hyperglycaemia, monogenic autosomal dominant mode of inheritance, age at onset less than 25 years, and the lack of auto-antibodies. Mutations in six genes have been described and these include the enzyme glucokinase, which causes MODY2, and the transcription

factors HNF-4 alpha, TCF1, Pdx-1, TCF2, and NeuroD1, which cause MODY1, 3, 4, 5, and 6, respectively. Most recently, KLF11 has been described as MODY7 and is a novel p300-dependent regulator of Pdx-1 (MODY4) transcription in pancreatic islet β -cells [11]. One thing that all these genes have in common is their expression in the mature β -cell.

Pdx-1 is a critical transcription factor in the adult β -cell and haplodeficiency in mice and humans leads to diabetic phenotypes. A recent study by Stoffers research group has highlighted an important new role for Pdx-1 in the regulation of β -cell endoplasmic reticulum stress responses, where it directly regulates a number of these genes and processes [37].

Recent genome wide association (GWA) scans of the human genome have identified over 20 new genes or genomic sites associated with type II diabetes [43]. A number of these genes are also expressed and act in the insulin-producing β -cell, with TCF7L2 being the best characterized [16, 28]. In addition, mutations in SLC30A3 and ZnT8, both which affect zinc transport, an important part of the insulin secretory granule, have been identified and shown to have important roles in β -cells [32, 40].

These data further highlight the complexity of the pancreatic β -cell and how precise regulation of the β -cell is so important especially under physiological stress such as diabetes.

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Chapter 5

The Role of the Cytoskeleton in Transport and Release of Insulin-Containing Granules by Pancreatic β -Cells

Roger S. Goody and Hans Georg Mannherz

Abstract Insulin secretion by β -cells is stimulated by a rise in blood glucose level and occurs in two phases: a first phase of short duration leading to the release of a small number of insulin-containing granules and a second phase lasting up to several hours. During the first phase primed insulin granules constituting the ready releasable pool (RRP) are exocytosed, whereas during the second phase this RRP is constantly replenished by granules from the reserve pool. During replenishment insulin granules have to be transported from more central intracellular locations towards the exit sites on the plasma membrane. Microtubules and the motor protein kinesin perform the long-distance transport of insulin granules; subsequently the motor protein myosin Va accomplishes their transfer along short F-actin filaments to the docking sites at the plasma membrane, where the granules are tethered by the formation of a ternary complex of Rab27a and granuphilin residing on the granular membrane and syntaxin 1a on the plasma membrane, leading to membrane fusion and insulin secretion.

Keywords Actin · Insulin · Kinesin · Microtubules · Myosin Va · Rab-GTPases

5.1 Introduction

The pancreatic islets are clusters of endocrine cells that secrete a number of different peptide hormones. The islets were first discovered in 1869 by Paul Langerhans (Berlin, Germany) and are named in his honour “Langerhans islets”. It was 20 years later that Oskar Minkowski and Joseph von Mering (Strasbourg, then Germany) induced diabetes mellitus in dogs by removing the pancreas. Early attempts to treat

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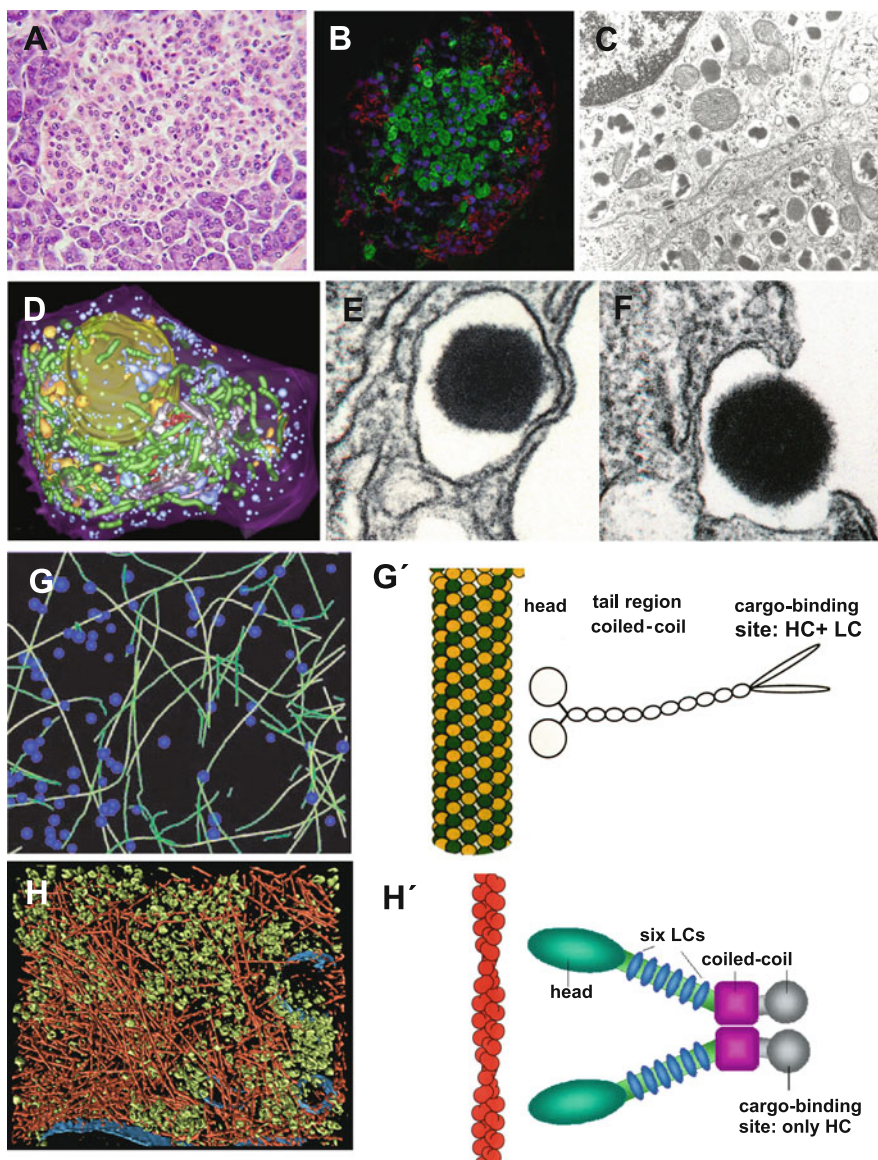


Fig. 5.1 Images of pancreatic islets and β -cells. Pancreatic islet visualised by the classical hematoxylin-eosin staining (a) and immunostaining with anti-insulin antibody (b). Electron microscopical image (transmission electron microscopy) of two adjacent β -cells (c) and after electron tomography (d) given in green the mitochondria and in blue the secretory granules (according to Marsh et al. [18]). (e) Single insulin granule docked to the plasma membrane and (f) during discharge. (g) Part of a β -cell showing MTs (green) and insulin granules (blue) in close proximity. (g') A schematic representation of an MT with protofilaments, which are built by the linear aggregation of tubulin heterodimers and an attached kinesin molecule (HC = heavy chain and LC = light chain). (h) An electron tomography images of the cortical web of a fibroblastic cell (according to Baumeister) with attached small secretory granules. (h') Schematic representation of an F-actin filament with an attached myosin Va molecule (for details see text).

diabetic patients with pancreatic extracts failed (Zülzer, Berlin, Germany). In 1916 Paul Paulescu (Bucharest, Romania) for the first time prepared a pancreatic extract enriched in insulin that he used successfully to treat diabetic dogs. Further work in his laboratory was held up by the First World War, and it was not until August 1921 that his results were published. In 1921 Banting and Best (Toronto, Canada) essentially repeated the results of Paulescu, and in 1922 they treated diabetic patients for the first time successfully using their highly insulin-enriched pancreatic extract. Among their first patients was a 5-year-old diabetic boy who, under continuous insulin treatment, lived till the age of 76 (see also [1]).

The human pancreas contains about one million dispersed Langerhans islets composed of five different cell types (A, B, D, E, or α , β , δ , ϵ , and PP), each specialised for the synthesis of a particular peptide hormone. With 65–80% the insulin-secreting B- or beta (β -) cells are the most abundant islet cell type (Fig. 5.1a, b). Insulin is the sole hormone that lowers the concentration of blood glucose. Therefore, defects in its release will invariably lead to the metabolic disease diabetes mellitus. Insulin secretion by β -cells is stimulated by an increase in blood glucose and usually occurs with a biphasic time course, i.e. rapid initial and a slow but sustained second phase. Because diabetes type 2 is characterised by the absence of the first phase and a reduction of insulin release during the second phase, understanding the cellular mechanism of the biphasic insulin secretion and its disturbance is of paramount importance.

5.2 Models to Study Insulin Secretion

Insulin secretion by β -cells has been studied in whole rodent pancreas preparations, isolated rat or mouse islets or primary β -cells. The response of these organ-typical preparations or primary cell culture systems to glucose and other secretagogues may correspond most closely to their *in vivo* behaviour. However, after isolation they survive only for a short period of time. Therefore attempts have been made to obtain and to recapitulate the data obtained from animal models with clonal or established β -cell lines, because they offer the advantage of propagation in cell culture and ease of handling. These cells can be stimulated by glucose to release insulin; however, their response is in most cases not clearly biphasic, although short and sustained insulin release responses can be evoked by modulating the external glucose concentration.

5.3 Metabolic Effects of Glucose in β -Cells

After nutrient uptake, the blood glucose increases. β -cells take up glucose by facilitated diffusion catalysed by the GLUT2 transporter. Intracellularly, glucose is metabolised by glycolysis, leading to an increase of the ATP/ADP concentration ratio. Increased ATP inhibits the ATP-sensitive K^+ channel resulting in an intracellular K^+ increase and membrane depolarisation that subsequently opens voltage-gated

Ca^{2+} channels. The increase in cytosolic Ca^{2+} ions is supposed to be the main trigger for exocytosis of the insulin-containing granules. A number of other secretagogues like KCl, cAMP and IBMX (isobutyl-methylxanthin, an inhibitor of PDEs) induce only a short response. They do not elicit the metabolic effects of glucose, in particular the ATP increase necessary for sustained insulin release (for a review see also [21]).

5.4 The Response of the β -Cell

As an exported protein, pre-proinsulin is synthesised by ribosomes attached to the rough endoplasmic reticulum (rER) of the β -cells as a single polypeptide chain (pre-proinsulin) of 110 residues including the N-terminal signal sequence, which is removed within the rER generating proinsulin of 84 residues. Enclosed in small vesicles, proinsulin is transported from the rER to the Golgi complex. Within the trans-Golgi network (TGN) proinsulin is processed to mature insulin by excision of the connecting peptide to generate the A-chain of 21 residues and the B-chain of 30 residues. The chains are connected by two disulphide bridges. During package in secretory granules by the TGN, mature insulin is complexed with Zn^{2+} ions, inducing inactivation and aggregation (of Zn^{2+} -containing insulin hexamers) to the crystalline dense cores within the insulin granules visible in electron microscopic images (Fig. 5.1c).

The cytoplasm of each β -cell is filled with more than 10,000 insulin-containing vesicles or dense-core granules (Fig. 5.1d), which are released by regulated exocytosis (Fig. 5.1e, f). Under resting conditions (fasting) the β -cell has to block the release of insulin-containing granules in order to secure a low blood insulin level. After stimulation only a small fraction of these granules release their content by exocytosis into the extracellular space around the β -cells, from where the released insulin rapidly diffuses into adjacent capillaries. Nutrient uptake, especially the increase in blood glucose level, stimulates β -cells to exocytose insulin-containing granules. During exocytosis the granular membrane fuses with the plasma membrane finally leading to fusion and subsequent fission of both membranes and release of the granular content into the extracellular space.

The elevation of blood glucose induces a biphasic insulin release: a rapid initial and transient phase lasting only a few minutes and a second sustained release up to several hours depending on the duration of the blood glucose elevation. The rapid first phase is characterised by the release of a relatively small amount of insulin from granules, which are docked in close apposition to the plasma membrane (see Fig. 5.1e) and already “primed” for discharge (see also Fig. 5.1f). Subsequently, the insulin secretion returns almost to the resting level. However, after nutrient uptake the blood glucose level usually remains elevated for longer periods of time and initiates the second sustained phase leading to the release of a larger amount of insulin.

It has been estimated that during the first phase only about 50 “primed” insulin granules are exocytosed. These are only a fraction of the granules visualised by morphological methods to be “tethered” to the plasma membrane. It has been suggested that the “primed” state of the granules is due to direct complexation to the voltage-gated Ca^{2+} -channel protein [2]. In contrast, during the second phase about 1,000–2,000 granules are transported from more distant regions of the cytoplasm towards the plasma membrane and discharged. Thus, stimulated β -cells are able to mobilise two different pools of insulin-containing granules: a readily releasable pool (RRP) of about 50 immediately releasable primed granules and a few thousand (about up to 2,000) granules of the so-called reserve pool that have to be either primed or translocated from more central cytoplasmic regions to the plasma membrane.

5.5 The Intracellular Cytoskeleton

The intracellular cytoskeleton appears to be responsible for both the inhibition of exocytosis during the resting phase and the active transport of insulin granules necessary for sufficient insulin release after stimulation. Principally the cytoskeleton is composed of three more or less independent filamentous systems: (i) the actin-containing microfilaments (MFs) and their associated proteins, (ii) the microtubules (MTs) composed of the α,β -tubulin heterodimer, and (iii) the intermediate filaments, which almost exclusively fulfil mechanically stabilising functions and will not be further described in this chapter. Specific motor proteins interact with either microfilaments or microtubules and are involved in the various forms of cellular motility. Motor proteins of the myosin family interact with microfilaments to generate force and kinesins and cytoplasmic dyneins perform transport processes of intracellular vesicles along microtubules.

5.6 Some Basic Properties of Microtubules

Cytoplasmic microtubules are long, straight filamentous structures with a diameter of 23–25 nm (Fig. 5.1g). They are composed of α,β -tubulin heterodimers (molecular mass: 2×55 kDa), which associate head-to-tail to long protofilaments. Thirteen protofilaments associate laterally to a closed tube, the microtubule. Microtubules (MTs) are polarised, possessing two different ends with different affinities for the tubulin heterodimer (Fig. 5.1g). Tubulin molecules preferentially associate to the so-called plus-ends. The closely related α - and β -tubulin subunits have a molecular mass of about 55 kDa and firmly bind one molecule of GTP. The GTP bound to β -tubulin is exchangeable and hydrolysed to GDP during the polymerisation process, whereas the GTP bound to α -tubulin is not hydrolysed and exchanged. Addition of tubulin heterodimers to the plus-end generates a so-called GTP cap, since GTP hydrolysis by the β -tubulin molecules occurs with a time lag. MTs with

a plus-end GTP cap are stable, but once this GTP is hydrolysed to GDP rapid shrinkage of the microtubule from this end occurs (see also [5, 20, 26]).

Intracellularly, the MTs originate from the centrosome, also termed microtubule organisation centre (MTOC), which is located close to the cell nucleus, and extend to the cell periphery. Their minus-ends are tagged into the MTOC probably by association with a specific tubulin molecule (γ -tubulin). Their plus-ends are located peripherally where their tendency to shrink is reduced or blocked by binding of plus-end capping proteins.

Single cytoplasmic MTs extend from the cell centre to its periphery and form the tracks along which vesicles can be transported over long distances in both directions. Specific motor proteins attached to vesicles or intracellular organelles crawl along the surface of MTs and thereby translocate vesicular structures either centripetally or centrifugally. The main representatives of MT-associated motor proteins are members of the kinesin and dynein families. These motor proteins associate with the vesicular membrane and move these in a processive manner along MTs. Most kinesins (Fig. 5.1g') translocate their cargo (vesicles) from the minus- towards the plus-end (centrifugally), whereas the cytoplasmic dyneins transport in the opposite direction [11, 14].

5.7 Conventional Kinesin Transports Insulin Granules During Second-Phase Secretion

The second phase of glucose-stimulated insulin secretion can last for several hours. It has been estimated that 5–40 insulin-containing granules are released per cell and minute during this phase [2]. Therefore insulin granules from the so-called storage or reserve pool located more centrally within the cell have to be translocated to the peripheral release sites.

It has been demonstrated that highly specific MT-disrupting drugs like colchicine or nocodazole block the second phase without affecting the initial fast phase of insulin secretion [8]. Given the direction of their movement, it appears plausible that members of the kinesin family transport the insulin granules along MTs [27].

Kinesins are elongated heterotetrameric proteins composed of two heavy and two light chains. The heavy chains form the three main structural domains: the two N-terminal globular heads followed by an α -helical coiled-coil and finally two tail regions with the attached light chains (Fig. 5.1g). The head regions form the highly conserved motor domains, each containing an ATPase centre and MT-binding site. ATP hydrolysis drives the force generating power stroke when attached to an MT. The tail regions function as cargo-binding domains whose interaction with vesicular membranes is mediated by specific adaptor proteins located on the cytoplasmic face of the cargo vesicles.

Kinesins spend a large fraction of the ATPase cycle attached to the MTs and are processive motor proteins. Processivity is further supported by the fact that a head

once bound to an MT does not dissociate before the second head has attached to the next binding region towards the plus-end of the MT. Thus the two heads of a kinesin molecule can perform a large number (around 100) of alternate ATPase and translocation cycles leading to migration of the whole kinesin along the MT in a hand-over-hand fashion, thereby transporting attached vesicles over long distances.

The kinesins are a large family of motor proteins and most eukaryotic cells express several kinesin variants, which fulfil specific transport functions determined by their cargo-binding sites. It has been shown that the so-called conventional kinesin heavy chain or kinesin I can be detected on isolated insulin granules of established β -cell lines [27]. Furthermore, transfection of β -cells of established lines with a dominant negative mutated kinesin or kinesin I-specific siRNA results in a clear reduction of the intracellular movement of insulin-containing granules and second-phase insulin secretion after sustained glucose stimulation [27]. These data indicate that the kinesin-dependent insulin-granule transport functions to replenish the readily releasable pool.

Kinesin activity depends on the intracellular ATP concentration, which is increased after glucose stimulation of β -cells. The higher the external glucose concentration, the higher is the elevation of the intracellular ATP concentration. Indeed, usage of permeabilised clonal β -cells demonstrated that the speed of insulin-granule movement correlates with the externally added ATP concentration. Thus, the speed of replenishment of the readily releasable pool is modulated by the external glucose concentration [27].

5.8 Some Basic Properties of F-Actin Filaments

The basic building blocks of the actin cytoskeleton are the microfilaments composed of actin subunits (Fig. 5.1h). The cytoskeleton, and in particular the microfilament system, is a highly dynamic system; it is constantly remodelled according to the cellular needs. A high fraction of the intracellular actin is maintained in monomeric form; this reserve pool of globular (G)-actin is used for the constantly occurring reorganisation of the actin cytoskeleton.

Monomeric G-actin has a molecular mass of 42 kDa and contains firmly bound one molecule ATP, which is hydrolysed into ADP and inorganic phosphate (Pi) after polymerisation and incorporation into an actin filament (F-actin). Whereas the Pi is rapidly released, the ADP remains firmly attached to F-actin subunits generating two different filament ends: the fast-growing plus or barbed end containing exposing ATP-actin subunits and the slow-growing minus or pointed end with ADP-actin subunits. The different filament ends are the basis for the polarised addition and dissociation of subunits to F-actin filaments: ATP-actin subunits attach to the barbed ends and ADP-actins dissociate from the pointed ends. After dissociation the actin-bound ADP is exchanged for ATP rendering it able to re-associate at the barbed end. The process that under steady-state conditions a single actin subunit travels after association to the barbed end through the whole filament before it dissociates from the minus or pointed end is termed treadmilling [31]. In motile cells a thin

veil-like extension of the plasma membrane (the lamellipodium) actively protrudes in the direction of cell migration. The lamellipodial forward movement is achieved by the continuous addition of new actin molecules to a branched F-actin network, whose barbed ends are oriented to the cell periphery [15].

In many tissue cells, including β -cells, an F-actin network is to a large part concentrated immediately underneath the plasma membrane forming the so-called cortical web (Fig. 5.1h). This network of short F-actin filaments is attached to the plasma membrane at multiple points by proteins of the so-called ERM (ezrin/radixin/moesin) family, which link it to particular cell adhesion molecules or extracellular matrix receptors like the integrins [25]. As in motile cells the barbed end of the F-actin network of β -cells is supposed to be oriented towards the cell periphery.

5.9 The Role of the Actin Cytoskeleton During Exocytosis

Although considerable knowledge about the function and regulation of the microfilament system has been accumulated in recent years, its exact role in exocytotic and especially in insulin-secreting β -cells is still not completely understood due to the fact that it appears to simultaneously fulfil a number of diverse functions. Staining of β -cells with TRITC-phalloidin demonstrated the existence of the above-mentioned dense network of F-actin filaments underneath the plasma membrane. It has been demonstrated that glucose entry into β -cells induces an immediate reorganisation of the cortical F-actin net into shorter filaments [29].

It was suggested that this cortical web physically blocks the access of insulin-containing granules to the plasma membrane in resting β -cells and furthermore might also impede their discharge after stimulation unless disassembled or reorganised. This assumption gained support from data showing that F-actin-disrupting drugs, especially the highly specific latrunculins, induce an increased insulin discharge during both phases after glucose stimulation [23]. However, latrunculin exposure did not induce insulin release of unstimulated cells [19, 23].

Therefore proteins with F-actin-fragmenting activity were suspected to aid insulin exocytosis after stimulation. These include gelsolin and the closely related scinderin [3], which is, however, expressed only in very low amounts in β -cells. Gelsolin fragments (severs) F-actin filaments after Ca^{2+} -ion activation [32] and its possible role in insulin secretion was repeatedly assessed by gelsolin siRNA-knock-down or comparing clonal β -cells differing in its expression [23]. Gelsolin “minus”-cells exhibited longer F-actin filaments, which in contrast to gelsolin-normal cells were not depolymerised after glucose stimulation, indicating a crucial role for gelsolin in the normal stimulus-secretion pathway. Consequently, these β -cell variants exhibited a reduced insulin release after stimulation that was, however, considerably increased after latrunculin exposure [23]. In summary, these authors concluded that efficient insulin release by stimulated β -cells necessitated depolymerisation of the cortical F-actin in order to accomplish fast and efficient replenishment of the RRP from the reserve pool [23].

This account cannot represent the whole story, since it was observed that latrunculin or other F-actin fragmenting manipulations (like transfection with *Clostridium botulinum* C2-toxin) in particular in clonal β -cells containing only a low number of insulin granules induce an inhibition of insulin release [16]. Therefore additional functions appear to be performed by the microfilament system.

5.10 Myosin Va and F-Actin Are Necessary for the Final Delivery of Insulin Granules to the Plasma Membrane

Kinesin transports insulin granules towards the plasma membrane; however, the final step of their transport to the release sites is performed by the interplay between F-actin filaments and the motor protein myosin Va. Proposals suggesting the involvement of myosins in secretory vesicle transport were made long before the discovery of MT-associated motor proteins [16, 17]. In contrast to conventional myosins II as supposed in these early suggestions, the analysis of the myosin V-deficient mouse model “dilute” clearly demonstrated the general involvement of the unconventional myosin Va in intracellular vesicle transport. Diluted mice have a post-natal life expectancy of only a few weeks due to a variety of neurological defects; their most obvious phenotype is the impaired transport of melanosomes and delivery to keratinocytes and hairs leading to reduced (diluted) fur pigmentation [9].

Myosin Va molecules are composed of two identical heavy chains ($M_r = 215$ kDa), which dimerise in their coiled-coil domain (Fig. 5.1h'). The N-terminal motor domains are followed by long α -helical shafts (lever arms), which are stabilised by six light chains per shaft – four of which are Ca^{2+} -ion binding calmodulins. Subsequently, the α -helical shafts unite to the coiled-coil domain, which finally forms two separate globular cargo-binding (vesicle) domains ([4]; see Fig. 5.1h'). A number of particular properties make myosin Va motors ideally suited for vesicular transport: (i) they bind to both MTs and F-actin; therefore, secretory vesicle can be equipped simultaneously with both kinesin and myosin Va; (ii) in contrast to conventional myosins they spend a large fraction of the ATPase cycle attached to F-actin (“high duty rate”); and (iii) they are processive motors performing multiple large steps (36 nm each) due to their long lever arms towards the barbed end of F-actin [22]. Recent data demonstrated that myosin Va is a component of the insulin-granule membrane [28], indicating that during second-phase secretion the final step of granule transport from the reserve pool to the release sites on the plasma membrane also necessitates F-actin.

5.11 Control of Granule Docking

In contrast to neurons, there are no defined release sites for secretory granules in endocrine cells and the slow (rate-limiting) process of insulin-granule docking has been made responsible for the release of low amounts of insulin after stimulation. Selective intracellular docking and fusion of vesicles is controlled by a particular

group of small GTPase proteins, the Rab-GTPases or Rab proteins (ras-like proteins from brain). There are more than 60 Rab proteins in human cells involved in specific vesicle-membrane targeting. The Rab proteins also indirectly control the association of myosin Va with insulin granules as well as melanosomes and other secretory vesicles. In melanocytes, this is quite well understood and involves the interaction of Rab27 with melanophilin, which in turn interacts with myosin Va. Interestingly, this interaction only occurs in the peripheral dendritic region, whereas the transport from the perinuclear region probably occurs via interaction of Rab proteins with kinesin and motion on MTs after their initial concentration near the MTOC by the interaction of Rab7 with the dynein motor system.

Rab proteins also direct the process of tethering, i.e. the initial contact of a vesicle with a target membrane, additionally supported by specific tethering factors, which are either coiled-coil or multimeric protein complexes. A mutation leading to functional deficiency of Rab27a in mice (ashen mice) leads to pigmentation disorder and to decreased insulin release after β -cell stimulation [30]. In β -cells, Rab27a is attached to the cytoplasmic face of the insulin granules and associated with Slac2-c and additionally with granuphilin, a molecule related to melanophilin. The Rab27a–granuphilin complex mediates the tethering of the granules to the plasma membrane by binding to Munc18-1 and syntaxin 1a [24]. Thus, membrane-specific tethering is mediated by Rab27a, which forms a ternary complex with granuphilin and syntaxin 1a. However, the thus tethered insulin granules are not yet primed and supposed to be even release-incompetent awaiting the activation of the fusion machinery [10]. A further Rab 27 effector (i.e. protein which binds to the GTP form of Rab 27) found on secretory granules is MyRip, which also interacts with myosin Va. Apart from Rab27a several other Rab proteins have also been implicated in insulin secretion [6, 7, 12].

Scaffolds

Scaffolds are permanent or temporary structures that support the construction of complex structures, or serve as a track for a transport system. In a sense, a ribosome is a scaffold for the assembly of proteins from dissolved peptides, by reading a code from an mRNA punch tape, and molecular motors like the cargo moving kinesin are using microtubules as a reversible scaffold to define the transport direction. Artificial biomimetic scaffolds may serve as a base for growing new tissue, e.g. bone or skin, pancreas or liver, to be degraded after tissue formation (Baptista PM et al (2009) Whole organ decellularization – a tool for bioscaffold fabrication and organ bioengineering. Conf Proc IEEE Eng Med Biol Soc 2009:6526–6529).

Added by the editors

5.12 Control of Granule and Plasma Membrane Fusion by F-Actin

Exocytosis requires the fusion of two separate unit membranes, the vesicle and plasma membrane, and the subsequent fission to allow the discharge of the vesicular content (see Fig. 5.1f). These processes are catalysed by the interaction of two sets of membrane proteins – the SNARE proteins (= soluble *N*-ethylmaleimide-sensitive attachment receptor). In mammalian cells there are more than 20 SNARE proteins, which convey specificity to membrane fusion events. In addition, the Rab proteins bound to the vesicular membrane further control the specificity of the membrane interactions. The largely α -helical cytoplasmic domains of both vesicular v-SNARE and target membrane t-SNARE interact with each other under the formation of highly stable coiled-coils in order to bring the two separate membranes in close apposition (docking), fusion and finally fission. The molecular details of these processes are not yet completely understood, but it is known that the energy for this process is delivered by the final dissociation and reactivation of the entangled SNAREs, induced by the ATP-consuming chaperon-like NSF protein. The insulin granule v-SNARE has been identified as the vesicle-associated membrane protein 2 (VAMP2) and the plasma membrane t-SNARE as syntaxin-4, which appears to be involved at least in the second-phase insulin release.

t-SNAREs are often blocked by binding of inhibitory proteins in order to avoid indiscriminate fusion events. Surprisingly, it was found that the inhibitory protein for syntaxin-4 is F-actin that specifically interacts with two of its α -helical domains (Jewell et al. 2008). This interaction is disrupted after glucose entry into the β -cell probably being part of the then initiated F-actin reorganisation. These events not only relieve the apparent physical barrier of the F-actin cortical web to the transfer of granules from the reserve pool to the plasma membrane, but more specifically allow the protein–protein interactions necessary for granule discharge.

Very little is known so far about the signalling pathways, which after glucose entry lead to microfilament reorganisation. It has been shown that the Rho-family GTPase proteins Cdc42 and Rac1 are transiently activated immediately after glucose entry. It will be interesting to identify their effector proteins responsible for the reorganisation of the F-actin cortical web in insulin-secreting β -cells.

5.13 Summary

Insulin-granule transport is a dual process depending on microtubules for long-distance transport and F-actin for the final delivery to the release sites at the plasma membrane. In addition, F-actin appears to fulfil multiple functions before and after β -cell stimulation that necessitate tightly controlled reorganisation events of its supramolecular organisation, which are far from being fully apprehended.

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Chapter 6

The Mathematical Microscope – Making the Inaccessible Accessible

Johnny T. Ottesen

If you want to learn about nature . . . it is necessary to understand the [mathematical] language that she speaks in
Richard P. Feynman

Abstract In this chapter we introduce a new term, the “mathematical microscope”, as a method of using mathematics in accessing information about reality when this information is otherwise inaccessible. Furthermore, we discuss how models and experiments are related: none of which are important without the other. In the sciences and medicine, a link that is often missing in the chain of a system can be made visible with the aid of the mathematical microscope. The mathematical microscope serves not only as a lens to clarify a blurred picture but more important as a tool to unveil profound truths. In reality, models are most often used in a detective-like manner to investigate the consequences of different hypothesis. Thus, models can help clarify connections and relations. Consequently, models also help to reveal mechanisms and to develop theories. Case studies are presented and the role of mathematical modelling is discussed for type 1 and type 2 diabetes, depression, cardiovascular diseases and the interactions between the combinations of these, the so-called grey triangle in the metabolic syndrome.

Keywords Mathematical modelling · Diabetes · Depression · Cardiovascular regulation · Systems biology · Measurements · Experiments

6.1 Introduction

With the use of mathematical models, it is possible to simulate almost any kind of phenomena in nature on a computer. This is a scientific practice that is

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presently permeating almost all areas of modern science, e.g. in biology, physiology, medicine, climate research, and ecology to name a few. Models serve a variety of purposes from prescribing what will happen in the (near) future to constituting the formation of theoretical concepts within the fields. They are used to replace costly and uncertain monitoring and to develop new monitoring equipment, e.g. the mathematically founded medical imaging method employing the tomography known from CT scanners. Models play a special role in designing new methods of measurements and they are crucially involved in all non-trivial measurements.

Research using mathematical modelling in medicine has become so important that this type of research now has its own name: *in silico*, which is analogous to *in vivo*, *ex vivo* or *in vitro*. The significance of mathematically based computer models has reached a level such that mathematical modelling undoubtedly will become the paradigm of scientific and medical research in the twenty-first century.

When scientific practices change, there is a need to pause and reflect upon how the new scientific practices should be pursued, what kind of insights they give, and to what degree and under what circumstances the new forms of knowledge can be trusted. These are urgent questions that need to be discussed in the scientific literature, especially considering the growing use of mathematically based computer models for decision support and planning tools in all fields of medicine.

This chapter focuses on mechanisms-based models and describes their actual underlying mechanisms, as well as on models used for extracting measurements in experiments. In research, the ultimate goal is to develop mechanisms-based models, but in reality models are more often used in a detective-like way to investigate the consequences of different hypotheses. Consequently, models help clarify connections and relations quite similar to how Sherlock Holmes uses logic to unravel crimes in the novels by Sir Arthur Conan Doyle. As a result, models help to reveal mechanisms and to develop theories.

This type of detective-like use of mainly mechanisms-based models primarily for experiments is the core topic of this chapter. We term these kinds of models and their use “the mathematical microscope”. In brief the mathematical microscope is a method where mathematics is used in accessing information about reality when this information is otherwise inaccessible or difficult to access. In this case, it is essential that the mathematics describe the underlying mechanisms, except for one aspect – the inaccessible part – and that the information in this part can be obtained indirectly from available data by use of the model only. The sequential chain model of the baroreceptor regulation of the human heart rate constitutes an example (the model is illustrated in Fig. 6.2 below). The part that takes place in the central nervous system is, roughly speaking, inaccessible, whereas all other links in the chain are sufficiently known. Hence, there is a missing link in the chain that might become visible with the aid of the mathematical microscope. Thus, the role of the mathematical microscope resembles that of the light microscope by making the invisible visible. As a result, the mathematical microscope serves not only as a lens that clarifies a blurry picture, but also as a tool that unveils profound truths.

6.2 The Mathematical Microscope

Harvey's Mathematical Microscope

Galenos of Pergamon (131–201 CE): All blood flows from the liver, and the heart, to all parts of the body where it is consumed.

William Harvey's arithmetical microscope (1616/1628):

- Heart capacity > 1.5 ounces
- Blood expelled per heart pump > 1/8 of heart capacity, i.e. >1/6 ounce
- Number of heartbeats > 1000 per half an hour
- Sum1: More than 10 pounds 6 ounces arterial blood are produced in half an hour
- Sum2: More than $\text{sum1} \times 48 = 540$ pounds of arterial blood are produced and consumed in a day
- Consequence: There must be capillaries.

Marcello Malpighi's light microscope histology confirmation (1661).

Further Reading:

Harvey W (1628) *Exercitatio anatomica de motu cordis et sanguinis in animalibus* [Anatomical studies on the motion of the heart and blood], Frankfurt a. M. 72p. English edition Harvey W (1993) *The Circulation of the Blood and Other Writings* (trans: Franklin KJ). Introduction by Dr. Andrew Wear. Everyman: Orion Publishing Group, London.

Added by the editors

Before making use of the mathematical microscope, we would like to reveal the historical case that inspired the author to introduce the phrase “the mathematical microscope”, namely the discovery of the closed circulation of blood by William Harvey (1578–1657).

Considered from a modern point of view, the ancient ideas of how the body functioned seem very odd. For thousands of years, these ideas have been robust and strongly coupled to religion. Before 1628, the concept of blood circulation as we know it today was not imaginable.

The Greek physician Galen or Galenius, who lived in the second century AD, spent most of his lifetime observing the human body and its functioning, especially by dissecting the bodies of dead soldiers and gladiators. Galen was inspired by Pliny the Elder, a Roman physician¹ who roughly believed that there were two distinct

¹Confusion about the nature of the heart, the blood and the role of the blood in the body had existed for centuries. Pliny the Elder, who lived from AD 23–79, wrote in a 37-volume treatise entitled *Natural History*, that “The arteries have no sensation, for they even are without blood, nor do they all contain the breath of life; and when they are cut only the part of the body concerned is paralyzed [...] the veins spread underneath the whole skin, finally ending in very thin threads, and they narrow down into such an extremely minute size that the blood cannot pass through them nor can anything else but the moisture passing out from the blood in innumerable small drops which is called sweat.”

types of blood. “Nutritive blood” was thought to be made by the liver and carried through veins to the organs, where it was consumed. “Vital blood”, which was thought to be made by the heart and pumped through arteries, was believed to carry the “vital spirits”.

Galen believed that the heart acted not as a pump, but rather that it sucked blood from the veins and that blood flowed through the septum of the heart from one ventricle to the other through a system of tiny pores. He did not know that the blood left each ventricle through arteries. Until 1628, the Galenic view of the body prevailed completely.²

These beliefs continued to be taught and were taken to be the unwavering truth until an English physician, William Harvey, challenged them in the late 1620s. A major reason for this is, of course, the fact that the tiny capillaries connecting the arterial side with the venous side of the cardiovascular system are far too small to be visible to the human eye.

Using a simple model, Harvey showed that the amount of blood leaving the heart in a minute could not conceivably be absorbed by the body and continually replaced by blood made in the liver from chyle. Unlike anyone before him, Harvey noted that the amount of blood forced out of the heart per hour far exceeded the total blood volume of the entire body. Stroke volume, which is the amount of blood ejected per heartbeat, was known to be approximately 70 centilitres or 0.07 litres. Since the heart beats 72 times per minute on average under normal circumstances, this corresponds to 121 litres per hour or 7.465 litres per day. This seemed absurd when compared to the blood volume, approximately 5 litres, the average person was known to have.

Consequently, this model-based evidence established the concept that blood must constantly move in a closed circuit, otherwise the arteries and the body would explode under the pressure. Based on this, Harvey announced the discovery of circulation. The reason for pointing this out in the present context is that Harvey discovered the circulation of blood approximately 50 years before the discovery of the light microscope³ and hence changed the world view by the use of a simple mathematical model.

The concept or method of using mathematical modelling, as a tool for making an inaccessible system accessible or an invisible system visible, is therefore being coined as “the mathematical microscope” in honour of William Harvey. Figuratively, this is illustrated in Fig. 6.1. We emphasize that the microscope, here depicted as a lens, is used as an analogy for the mathematical microscope.

²In many cultures, physicians, as well as ordinary citizens, had their own beliefs concerning the nature of the heart and circulatory system. While the Greeks believed that the heart was the seat of the spirit, the Egyptians believed the heart was the center of the emotions and the intellect. The Chinese believed the heart was the centre of happiness. Even today in Western culture, remnants of these beliefs can be found in various sayings, “a broken heart”, “follow one’s heart”, “sweetheart”, etc.

³Anton van Leeuwenhoek’s microscope from 1674 is considered to be the first functioning microscope. He was the first to see and describe the capillaries of the circulatory system.



Fig. 6.1 Harvey discovered the circulation of blood approximately 50 years before it became visual by the discovery of the light microscope. This was done by the use of a simple mathematical model. The concept or method of using mathematical modelling, as a tool for making an inaccessible system accessible or an invisible system visible, is coined as “the mathematical microscope” in honour of William Harvey. We emphasize that the microscope, here depicted as a lens, is used as an analogy for the mathematical microscope.

6.3 Models Are Crucial in Measurements and Experiments

The design of measurement protocols or experiments is always based on models just as the interpretations of the results are. However, models are more fundamentally involved in measurements and experiments than this. In every non-trivial measurement and experiment, the desired quantities are not measured directly but are derived from the use of models, e.g. in measuring the resistance of an ohmic resistor experimentally, which is typically done by measuring several related values of potential drop across and current through the resistor, and if these fall on a straight line in a current–potential plot, then the slope defines the resistance; hence, the resistance is calculated based on the model $U = RL$. Note that a hierarchic system of models enters into the problem; the equipment measuring the potential drop and current also depends on models. This kind of hierarchic system of involved models is usually the rule rather than the exception in experiments.

Sometimes, the phrase “all models are wrong” is used or even, though put more delicately, “models are no better than their input.” These statements encompass some truth, but are at the same time misleading. Put more concisely, one should say that “models are not in a one-to-one correspondence with reality.” It is crucial that all models neglect unimportant information on whatever is being modelled, where defining “unimportant” depends on the purpose of the model.

However, since non-trivial measurements and experiments cannot be performed without models, all measurements and experiments are equally wrong!

Theory–Model–Experiment: Towards a Classification

The following taxonomy of models may be extremely useful, not necessarily for the credibility of mathematical models, but for the way of checking the range of credibility.

Yuri Manin, one of the strongest contemporary mathematicians, subdivided the mathematization, i.e. the way mathematics can tell us something about the external world, into three modes of functioning:

1. An (*ad hoc*, *empirically based*) *mathematical model* “describes a certain range of phenomena, qualitatively or quantitatively, but feels uneasy pretending to be something more”. Manin gives two examples of the predictive power of such models, Ptolemy’s model of epicycles describing planetary motions of about 150 BCE, and the Standard Model of around 1960 describing the interaction of elementary particles, besides legions of *ad hoc* models which hide the lack of understanding behind a more or less elaborated mathematical formalism of organizing available data.
2. A *mathematically formulated theory* is distinguished from an *ad hoc* model primarily by its “higher aspirations. A theory, so to speak, is an aristocratic model.” Theoretically substantiated models, such as Newton’s mechanics, are not necessarily more precise than *ad hoc* models; the coding of experience in the form of a theory, however, allows a more flexible use of the model, since its embedding in a theory universe permits a theoretical check of at least some of its assumptions. A theoretical assessment of the precision and of possible deviations of the model can be based on the underlying theory.
3. A *mathematical metaphor* postulates that “some complex range of phenomena might be compared to a mathematical construction”. As an example, Manin mentions artificial intelligence with its “very complex systems which are processing information because we have constructed them, and we are trying to compare them with the human brain, which we do not understand very well – we do not understand almost at all. So at the moment it is a very interesting mathematical metaphor, and what it allows us to do mostly is to sort of cut out our wrong assumptions. If we start comparing them with some very well-known reality, it turns out that they would not work”.

Further Reading:

- Manin Y (2007) *Mathematics as metaphor: selected essays by Yuri I. Manin with foreword by Dyson FJ*, American Mathematical Society, Providence, R.I. pp 3–26
- Bohle–Carbonell M, Booß B, Jensen JH (1984) Innermathematical vs. extramathematical obstructions to model credibility. In: Avula X (ed) *Mathematical modelling in science and technology. Proceedings of the 4th International Conference (Zürich, August 1983)*, Pergamon Press, New York, NY, pp 62–65

Added by the editors

Thus, instead of such an unproductive war on catchphrases, we prefer to take the stance that measurements and models as well as models and experiments are intricately entangled. To bring matters to a head: (non-design) *models are useless without data – data cannot be generated without models*, which leads us to a kind of chicken and egg situation. Of course models required to generate data are not

necessarily the same models as those intended to be supported by the generated data. Moreover, it follows that dissociating the two or explaining how to escape this morass in general is not easy.

For the sake of completeness, we emphasize that models are used in a great variety of ways, which is why it is often beneficial to divide them further into different categories, e.g.: design models, where reality is constructed with the help of models, financial systems, credit card systems, tax systems, utility items, buildings, airplanes, etc.; prediction models, e.g. weather forecasts, tsunami warning systems, navigation, the capacity of an oil reservoir, climate models, etc.; ad hoc models, where the underlying mechanisms are not described or known, e.g. options and futures in finance, population models, etc.; and qualitative models, which serve as explanatory devices rather than exact quantitative resemblances, e.g. chaos models, stability models, models of solar systems and supernovas, etc. The list is long and most models belong to a mixture of several categories like the aforementioned ones.

In a clinical setting, measurements and experiments appear in research related to diagnosing and planning treatment. In this context, medical doctors need to take individual consideration. The ultimate form of expression for this kind of individual approach is based on patient-specific models. Patient-specific parameter estimation undoubtedly belongs to the future but it is partially possible today. It is an opportunity for the pharmaceutical industry and medical doctors to target causes instead of treating symptoms: Complex models of otherwise inaccessible parts and processes can be used for estimating parameters describing inaccessible parts and processes. As a result, individual and patient-specific measurements are performed indirectly with the help of models; hence, biomarkers can be obtained. These issues will be elaborated further in the next section.

6.4 What Insights Can Modelling Provide?

Pronounced reductionism is widespread in scientific and medical research. Experimentally complex systems are often subdivided into parts and studied separately. In biology and medicine, subsystems are not easily put back together and conclusions made concerning subsystems have limited validity for the original undivided system as their function and dynamics may have changed when isolated from one another. On the other hand, some parts of the body as a whole cannot be studied *in vivo*, since the parts may be inaccessible for ethical reasons. The human pancreas is an example of this kind of delicate organ.

Parts that cannot be isolated experimentally can be studied (separately) using modelling. Mathematics is able to unfold the influence that each of the processes has on the overall dynamic behaviour of a complex system: Modern experimental science – especially modern biology – is highly adept at separating systems into components simple enough for their structures and functions to be studied in isolation. Mathematical modelling is the *only controlled* way to put the pieces back together by using equations that represent the system's components and processes, as well as its structures and interactions.

In many medical contexts, concepts are vaguely defined or even ambiguously defined. These kinds of ambiguous definitions suit the flexibility needed in most practical clinical situations. However, the lack of strictly well-definedness of concepts is very problematic in the search for truth in research. Ultimately, it leads to inconsistencies and misunderstandings, e.g. when discussing the contractility of the heart or debating Starling's law of the heart, i.e. whether the preload determines the cardiac output or the cardiac output determines the preload. Thus, modelling is the only way to strictly define concepts well and the only way to obtain values for measureable quantities (in combination with experiments.) In addition, we emphasize that modelling is an outstanding tool for proposing new experiments that would hardly be possible without a model.

Apart from the necessity of all reliable models in physiology being based on solid knowledge, they must also be primarily based on the underlying mechanisms involved as well as adequate data. This kind of knowledge and extensive data material related to, e.g., diabetes and other pathologies do exist. Data material has to be investigated and essential knowledge extracted. Statistical methods such as approximated entropy (regularity statistics known from non-linear dynamics) and generalized principal component analysis may reveal further information that forthcoming models have to encompass.

Models should be developed so they incorporate the responsible mechanisms for the modelled phenomena, i.e. they must be mechanisms based and, in addition, they should be based on first principles (conservation laws, etc.) whenever possible. Thus, mechanisms-based models may be rather detailed models. Somehow in oppose to this demand but in order to identify and estimate patient-specific parameters in an effective and reliable way, the number of parameters has to be kept as low as possible, which means that any unimportant factors and elements should be excluded, i.e. the so-called principle of parsimony must be obeyed. Hence a compromise between these conflicting demands often results in models based on elements resembling the underlying mechanisms as well as lumped elements. In any case, all parameters should have physiological interpretations. We denote such models as canonical models.

Patient-specific models are canonical models (preferable mechanisms-based) with physiologically interpretable parameters related to different pathologies and healthy states in which the values of the parameters are individually estimated. Thus, patient-specific models are canonical models that can be adjusted to specific individuals. Hence, in patient-specific models, pathologies are clarified by the values of certain parameters. The parameters are estimated from measurements in combination with the model, thus giving rise to more precise clinical diagnoses and more reliable suggestions for treatments than are known based on today's practices. In addition, existing classes of diagnosed cases may be refined into subclasses of pathologies corresponding to the actual defect of the physiological system by use of such patient-specific models. Moreover, knowing the actual defect(s) makes the development of target-specific drugs and other treatments possible. Development of this kind can guide the pharmaceutical industry in its search for new and improved drugs. In addition, a huge reduction in the cost of developing new drugs may be

expected not only due to a more beneficial process when searching for drug candidates but also because models may be used to substitute some costly animal and human experiments in future pre-clinical and clinical trials, respectively. Notice that patient-specific models used for accessing otherwise inaccessible patient-specific parameters make use of the methodology of the mathematical microscope.

The parameters have to be estimated by statistically founded algorithms (e.g. the extended Kalman filter, the Nelder–Mead algorithm combined with simplex methods, multidirectional search, particle filter/sequential Monte Carlo methods, genetic algorithms, etc.) or by functional analysis, i.e. optimal control, functional differential analysis, collocation methods, etc. Not all the parameters will necessarily be identifiable due to limitations concerning available data and/or an over-parameterization of the model. Thus, the estimation process has to be an iterative procedure coupled with sensitivity analyses or generalized sensitivity analyses combined with subset selection strategies, for instance.

An important part of the validation process, i.e. lack of falsification, is to compare model results with data (ideally with data independent of the data set used to estimate parameters). Analysis of model reductions, analysis of variations of sub-mechanisms, analysis of possible stability and bifurcations, analysis of possible limit cycle behaviour, etc. are all supplementary validation methods. If a model fails to be validated, it needs to be adjusted, which often gives rise to new insights into the underlying physiology.

When well-validated models with patient-specific estimated parameters exist, the identification of potential biomarkers becomes achievable. Potentially parameter estimation by patient-specific models may identify windows for parameter values defining different states for patients, e.g. diseased or healthy. This would be a big step forward for health care compared with empirical developed biomarkers, since the former also pinpoint the pathological part of the system for diseased patients. When such potential biomarkers are identified, different groups of patients, i.e. pathological subjects versus non-pathological subjects, can be examined. Notice that some of the parameters between two different groups have to vary. To determine whether there is a “real” difference between the values of the parameters (i.e. the biomarkers) within two groups or whether suggested biomarkers can identify variant causes (i.e. pathologies) of the illness (diagnosed by symptoms), statistical tests have to be performed. The biomarkers will definitely give rise to a classification of variants of the illness because they have inherent features that mean they are naturally in accordance with data from clinical diagnoses.

6.5 Example 1: Cardiovascular Diseases

In relation to cardiovascular diseases, simple models have been used for many years, e.g. to measure blood pressure, to transform blood pressure measured with a finger plethysmography to a central blood pressure, to measure blood flow velocities using ultra sound, etc. However, the potential is exceedingly greater than is the case

today. One example is that of people experiencing syncope due to problems with their autonomic regulatory system. Syncope is the medical term for severe dizziness or fainting due to oxygen depletion and carbon dioxide accumulation in the brain caused by insufficient blood supply to the brain. Syncope may be provoked by standing up too quickly or postural changes in general. Thus, this could be investigated clinically using head-up tilt experiments in which at least blood pressure and the heart rate are measured. Forty percent of all people experience syncope at least once in their lives. Syncope is a prevalent disorder, accounting for up to 6% of all hospital admissions each year in the Western world.

In healthy humans, autonomic nerve activity is inaccessible for ethical reasons, but insight into this area can be achieved using a mathematical model. Take, for instance, baroreceptor regulation of the heart rate for simplicity. This type of regulation comprises a feedback system consisting of several links in a sequential chain. Figure 6.2 illustrates the elements of the feedback chain controlling heart rate.

When blood pressure changes, as it does within one heartbeat and in between heartbeats, the visco-elastic arteries dilate or contract, whereby the circumferential of the vessels changes. Baroreceptors are located inside the wall of the carotid sinus arteries located in the neck (and elsewhere). These nerves are sensitive to visco-elastic deformations. The cellular exchange of ions, such as sodium and potassium, of these nerves is governed by a system of single channels and the gates are sensitive to the aforementioned deformation of the cell. This causes spiking in the potential across the cell membrane, which in this case is called the firing activity of the baroreceptor nerves. This activity is transported through the afferent nerve path to the central nervous system. In the medulla oblongata, the signal may mix with other signals, such as the muscle sympathetic stimulation, the respiratory control signals, and the low pressure receptor signals. These signals result in two efferent signals, the sympathetic tone and the parasympathetic or vagus tone, obeying the so-called inverse law and the direct law, respectively. The two tones (nerve activities) travel in different efferent pathways, the sympathetic nerve path and the parasympathetic

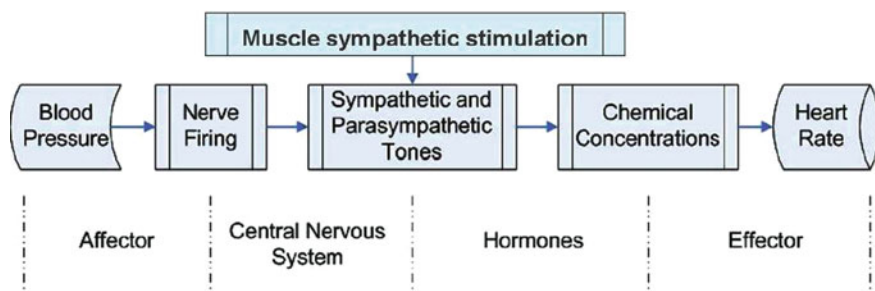


Fig. 6.2 Pulsatile blood pressure is the input to the baroreceptor nerves and it predicts baroreceptor firing rate. Sympathetic and parasympathetic tones respond in CNS to afferent activity and combines with muscle sympathetic stimulation. Sympathetic (delayed) and parasympathetic activity propagates to the synapses at the nerve endings where acetylcholine and noradrenalin are released. An action potential builds up at the sinus node in the heart and triggers it to beat.

nerve paths, respectively. The parasympathetic signal has a high transport velocity, whereas the sympathetic is five to seven times slower and is furthermore inhibited by parasympathetic activity. These pathways are spread out throughout the entire body and the parasympathetic tone affects vessel compliance, resistance, and the heart rate, whereas the sympathetic tone affects heart rate and contractility of the heart at a minimum. In fact, some substances like adrenaline and acetylcholine are released at the synapses at the nerve endings by these tones. The higher the activity, the more these substances are released. These substances take part in building up an action potential at the sinus node of the heart. When a threshold is reached, the heart beats and the action potential resets. For further details on this model, see [32–35, 39] and Olufsen et al. [27, 29, 30].

Note that the model goes from macroscopic pressure (millimetres) to cellular biochemistry (nanometres–picometres) and back to the organic level (centimetres). Finally, the heart affects the entire body (metres) if coupled to a cardiovascular model. Thus, the model is a multi-scale model; it scales from picometres over nanometres and centimetres to metres.

Hence, by doing an *in silico* examination of the feedback mechanism, the inaccessible parts become accessible. In our case, the methodology illustrates how access to the otherwise inaccessible separate links of the baroreceptor feedback chain regulating the heart rate can be obtained. As a result, insight into an individual's control system provides a “fingerprint” of the system, which may be of relevance for the treatment of various diseases such as hypertension (see Olufsen et al. [29, 30]).

It is currently investigated if such models can be used to generate biomarkers and thus categorizing the apparently different kinds of syncope. If so, the treatment will become differentiated, i.e. patient-specific or individual, and the medical treatment may intervene directly with the specific pathological part. This is in contrast to most treatment of today, which are merely treatment of symptoms.

6.6 Example 2: Type 1 Diabetes

Diabetes is a life-threatening condition. As stated in Chapter 1, more than 250 million people live with diabetes and the disease is associated with enormous health costs for virtually every society. It is estimated that diabetes is currently responsible for more than 6% of all deaths worldwide. Type 1 and type 2 diabetes are, roughly speaking, equal in number when counted in man-years, i.e. the number of diseased people times the average amount of years they have lived with the disease.

For a medical description of type 1 diabetes, see Chapter 1. Blasio et al. [6], Marée et al. [20–23], Jacobsen [14], and Nielsen [26] discuss a hierarchy of different but similar models. Each of these models describes the outbreak of type 1 diabetes, which is considered to be an auto-immune inflammatory process. Figure 6.3 encapsulates the essence of each of these models.

In Fig. 6.3, M denotes the amount of macrophages, M_a the amount of active macrophages, B the amount of β -cells, B_a the amount of apoptotic β -cells, B_n the

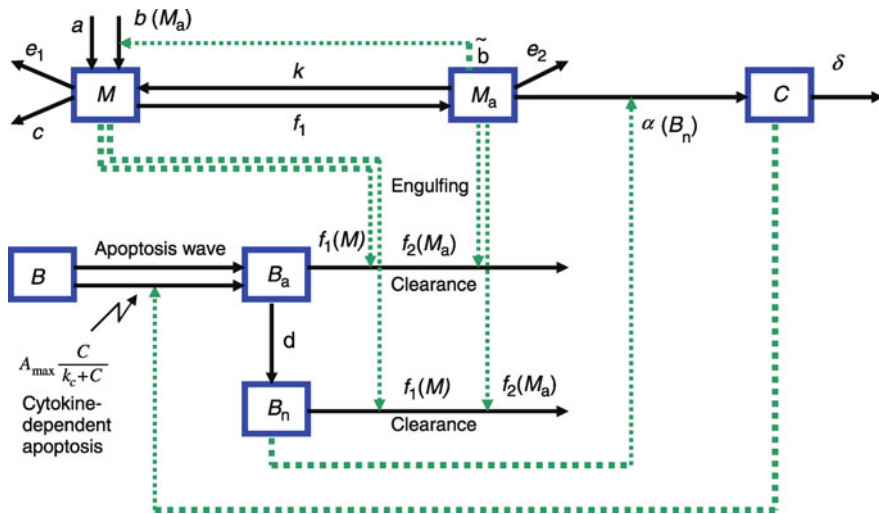


Fig. 6.3 Illustration of the compartment model describing development of onset of diabetes type 1. M denotes the amount of macrophages, M_a the amount of active macrophages, B the amount of β -cells, B_a the amount of apoptotic β -cells, B_n the amount of necrotic β -cells, and C the amount of cytokines produced during the engulfment of necrotic β -cells by active macrophages. Full arrows symbolize the flow and the rate is indicated nearby, e.g. the flow of macrophages becomes active f_1 times the instantaneous amount of macrophages, whereas the amount of cytokines produced per time unit is “ α ” times the instantaneous amount of necrotic β -cells and active macrophages. Dotted lines represent dependence, e.g. the above production of cytokines depends on the amount of necrotic β -cells. Thus, dotted arrows represent information only and correspond to processes in which the entries at the start of the dotted arrows are involved without being consumed by the process itself, similar to a catalytic process.

amount of necrotic β -cells, and C the amount of cytokines produced during the engulfment of necrotic β -cells by active macrophages. Full arrows symbolize the flow and the rate is indicated nearby, e.g. the flow of macrophages becoming activated is f_1 times the instantaneous amount of macrophages, whereas the amount of cytokines produced per time unit is “ α ” times the instantaneous amount of necrotic β -cells and active macrophages. Dotted lines represent dependence, e.g. the above production of cytokines depends on the amount of necrotic β -cells. Thus, dotted arrows represent information only and correspond to processes in which the entries at the start of the dotted arrows are involved without being consumed by the process itself, similar to a catalytic process. One main result is that if a certain combination of parameters is below a threshold value, the system is in the normal state, which is stable, but if the combination is above the threshold, then the normal state becomes unstable and two new stable states appear. The two new states are related to pathological states. In more refined models, several thresholds may exist characterizing transitions from one physiological state (pathology) to another. In the simplest case, where the cytokine-induced apoptosis is substituted by an apoptosis which is proportional to the number of active macrophages (i.e. where the dotted arrow $A_{\max}[C/(k_c + C)]$ – here k_c denotes the value of C where the effect is half the

maximal effect – going from the cytokine compartment to the cytokine-dependent apoptosis rate in Fig. 6.3 is substituted by a dotted arrow going from the active macrophages compartment having a fixed rate l , the system is in the healthy and stable steady state if:

$$\frac{f_1 l}{ck} < 1$$

and if not the system loses its stability, i.e. if the system drifts towards a pathological state. This qualitative prediction is confirmed by engineered mice. Non-obese diabetic mice (do not fulfil the stability criteria) develop type 1 diabetes under normal circumstance, whereas Balb/c mice (fulfilling the stability criteria) do not develop type 1 diabetes under normal circumstances. For humans, due to ethical considerations, we cannot access the parameters *in vivo* and even *in vitro* it is complicated. However, this result gives us insight into which parameters affect the multiple-caused outbreak of type 1 diabetes and in which combination.

6.7 Example 3: Type 2 Diabetes

In [1, 3, 4, 9–11, 16, 24, 42, 43, 46–48], the golden test for measuring insulin sensitivity is described and modelled: An artificially high insulin bolus is injected (generating hyperinsulinaemia), which consequently lowers the glycaemia. Glucose is then injected over a period of time to maintain normal glycaemia. Glycaemia and insulinaemia are measured during the test. Based on these measurements and a specific non-linear extension of the minimal model, data can be fitted and parameters estimated. Thus, the possible non-normal parameters pinpoint the specific pathology. It is, however, still work in progress to make a one-to-one correspondence between sets of non-normal parameters and, what then is, the corresponding different variants of type 2 diabetes, or more fundamentally whether type 2 diabetes is a collection of different pathologies characterized by the non-normal parameters in the model. If the answer is affirmative, such application of the model may serve as an excellent illustration of the mathematical microscope.

6.8 Example 4: Depression

Depression is a very common disease. Approximately 10% of people in the Western world experience severe depression during their lifetime and many more experience a mild form of depression. Endocrine pathologies are believed to be responsible for depression as well as for stress.

The HPA axis, see Fig. 6.4, is normally considered, by and large, as a self-regulated dynamic feedback endocrine system essential for maintaining body homeostasis in response to various stresses [7, 12, 15, 17, 18, 51]. Both physical and psychological stressors activate the hypothalamus to release corticotropin-releasing

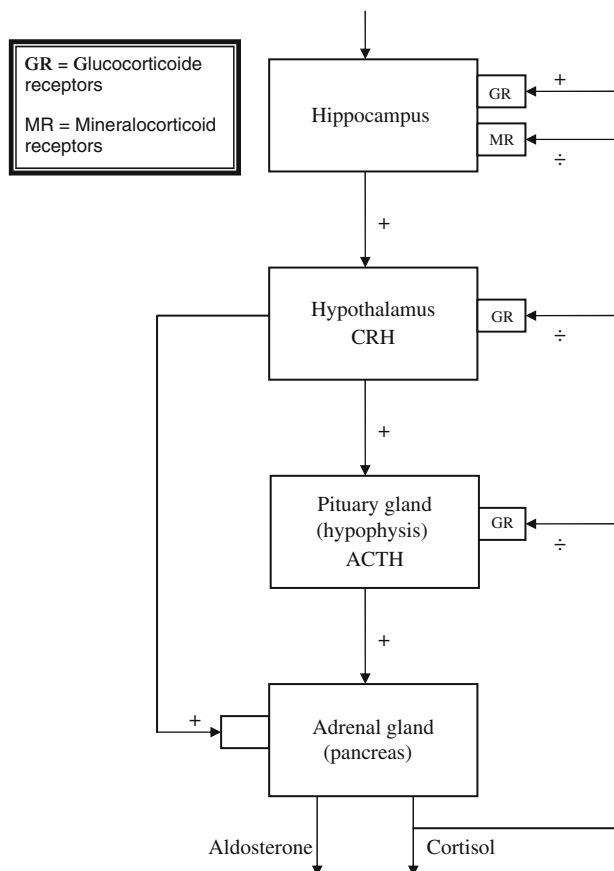


Fig. 6.4 The HPA axis is a self-regulated dynamic system. Stress activates the hypothalamus to release corticotrophin-releasing hormone (CRH). The CRH stimulates the pituitary to secrete adrenocorticotrophic hormone (ACTH). ACTH is released into the blood, where it is distributed throughout the body. The amount of ACTH flowing to the adrenal gland induces the synthesis and secretion of cortisol from the adrenal cortex. Cortisol has a feedback effect on the hypothalamus and pituitary GR = glucocorticoid receptor; MR = mineralocorticoid receptor.

hormone (CRH). The CRH is released into the closed hypophyseal portal circulation, stimulating the pituitary to secrete adrenocorticotrophic hormone (ACTH). ACTH is released into the blood, where it is distributed throughout the body. The amount of ACTH flowing to the adrenal gland induces the synthesis and secretion of cortisol from the adrenal cortex. Cortisol has a negative feedback effect on the hypothalamus and pituitary that further dampens CRH and ACTH secretion. This feedback is mediated by the blood circulation. Recently, it has been shown that the characteristic ultradian pulsatility observed in ACTH and the cortisol data cannot be reproduced by existing models for physiological realistic values of the parameters [2, 13].

The endocrine system usually shows regular oscillations, such as ultradian pulsatility ($\frac{1}{2}$ –2 h periods) and a diurnal cycle, in addition to oscillations with longer periods (which are not considered here). This is the case for the hypothalamic–pituitary–adrenal axis or the HPA axis, for instance. Recent research has shown that ultradian pulsatility cannot be explained by external factors (input from outside the body) nor by non-linearity or time delays in the known underlying bio-chemical reaction kinetics [2, 13]. Thus, these oscillations have to do with unknown mechanisms or couplings between the specific endocrine system and other subsystems in the body. It is a possibility that the coupling with the cardiovascular system causes some of these oscillations. We elaborate on this point in the next section. Another and maybe more likely possibility is that feedback processes in hippocampus and hypothalamus from the steps where cortisol attaches to the MR- and GR-receptors to the synthesis of CRH in hypothalamus take up to 18 min or more. If such a time delay is present, ultradian oscillations appear; however, until today such delay mechanism is not explicitly known.

In summary, the major result obtained is that all existing models made in accordance with state-of-the-art endocrine physiology are unable to generate the aforementioned ultradian oscillations observed in the ACTH and cortisol data unless one uses values far away from the physiological parameters. This result is a general mathematical result that only makes use of the fact that the feedback mechanisms of cortisol on CRH and ACTH production are limited by some saturation level.

6.9 Example 5: The Grey Triangle in the Metabolic Syndrome

Hormones are transported by the cardiovascular system and many hormones influence the cardiovascular system and its regulation, whereby a complex feedback system is established. Mathematical modelling with patient-specific parameter estimation is one of the most promising candidates for developing a general method for understanding oscillations in concentrations of hormones in the blood. Our future goal is to obtain knowledge about which oscillations the interaction between the endocrine system and the cardiovascular system may cause. Which factors are essential for these oscillations? What effects do the different states of the cardiovascular system have on the concentration of hormones in the blood and on blood sample measurements taken from the forearm vein? What effects do different disturbances of the hormone balance have on the oscillation pattern? And, finally, which oscillations, if any, can be generated by different (normo- as well as patho-) physiological parameter values?

Both ACTH and cortisol affect the cardiovascular system through multiple effects, thus redistributing the blood flow and thereby feedback on the HPA axis. Thus, the system encompassing the endocrine HPA axis and the cardiovascular system with autonomous regulation constitutes a complex dynamic multiple feedback system [12, 51], the sympathetic–adrenergic–medulla axis (SAM), which is part of the autonomic system that reacts to acute stress, and the slower reacting

HPA axis. The SAM axis prepares the subject for an immediate response by a sympathetic stimulation causing an increase in blood pressure and heart rate and release of catecholamines such as adrenaline and noradrenaline controlling the cardiovascular system [8, 28–31, 33–38, 40]. Here one expects the relevant regulated cardiovascular effectors to be those working on a timescale of $\frac{1}{2}$ –1 h.

Many diseases have a strong stress component. In this context, two physiological stress systems are often discussed: the SAM axis, which reacts to acute stress, and the slower reacting HPA axis. The SAM axis prepares the subject for an immediate response with a sympathetic stimulation causing a release of catecholamines such as adrenaline and noradrenalin resulting in an increase in heart rate and blood pressure. The HPA axis, on the other hand, releases the hormones corticotropin-releasing hormone, adrenocorticotrophic hormone, and cortisol [51].

There are direct links between the two systems. ACTH thus binds to the melanocortin-2 and -4 receptors and usually acts centrally to increase sympathetic nerve activation and cardiovascular tone [12]. The two systems furthermore counteract stress in a concerted fashion. Low SAM and HPA axis activities are seen in subjects able to cope with stress. In subjects unable to cope with stress, the two axes are permanently activated, and the collective system does not return to homeostasis. Under these circumstances, diseases like the metabolic syndrome, depression, burnout syndrome, and chronic fatigue syndrome may be the result.

Because both the SAM axis and the HPA axis show a large degree of plasticity, it can be extremely difficult to quantify the impact of an intervention or even the impact of the stressor by applying classical statistical methods. Consequently, there are many instances where new potential drug candidates fail to show their effect. In this respect, mathematical modelling of the two axes, including various feedback mechanisms and interaction mechanisms, might offer an advantageous alternative.

Modelling of the SAM and HPA axes and modelling of the interactions between the two systems are very important for the development of treatments for a number of diseases, of which only a few are mentioned here.

Other hormones which might be taken into consideration, if necessary, are insulin and glucagon hormones: Pancreatic islets release insulin and glucagon and smaller amounts of other hormones to the blood. Glucagon, released by alpha cells when glucose levels of the blood are low, stimulates the liver to release glucose in the blood. Insulin is released by β -cells when glucose levels of the blood (and amino acids) are rising. It increases the rate of glucose uptake and metabolism by most cells in the body.

It is well documented that corticosteroids increase insulin resistance in healthy people by 30–62% [5, 19, 25, 41, 44, 45, 49, 50]. In fact, one may inadvertently exacerbate stress hyperglycaemia through increased insulin resistance (see Fig. 6.5).

A non-pulsatile model of the cardiovascular system is sufficient due to the much larger timescale of interest than the period of time a heartbeat takes. The model has to include regions of relevance, e.g. head (and hypophyseal circulation), pancreas, heart (and lungs), liver, kidney, forearm (for measurements), and peripheral tissue (see Fig. 6.6). Relevant cardiovascular regulatory mechanisms,

such as the autonomic system (sympathetic and parasympathetic), autoregulation, myogenic effects, and in particular catecholamine and vascular tone, are believed to be essential.

In summary, stress and depression influence the cardiovascular system directly as well as indirectly through cardiovascular control processes. Furthermore, they influence the insulin sensitivity whereby the metabolic process is affected as well. In diabetic patients, one sees cardiovascular changes over time related to glycaemia. Finally, the cardiovascular system serves as a transport system for the hormones related to depression and diabetes. All three systems couple. Despite this interrelation, traditional research into and treatments for cardiovascular diseases, diabetes and depression are not coupled. In any case, developments as well as hurdles exist. Many of these hurdles and much misunderstanding may be due to the complex interaction between the three, e.g. what does the concentration of a hormone in the blood mean during various blood flow distribution conditions? Which oscillations are caused by coupling to other subsystems? The grey triangle refers to the interaction between the three.

Figure 6.6 illustrates a cardiovascular model that includes the most important interactions with the HPA axis and the insulin–glucose–glucagon system.

The state of the art in this field is now at a point where mathematical models can be used to examine the most important interactions between the cardiovascular system, the insulin–glucose–glucagon system, and the HPA axis. This type of research cannot easily be done on people. Only models allow the option of exploring different hypothesis under controlled circumstances.

6.10 Discussion and Conclusions

In physiology, there are problems in identifying the parameters of various systems. Some of them can be overcome by an intelligent combination of models and measurements. However, a generic problem remains: Often the effect of one subsystem on another subsystem comprises multiple effects operating through different pathways. Let subsystem A influence subsystem B along two different pathways, denoted x and y . Imagine that we control the input for subsystem A and are able to measure output from subsystem B, then, although it is, in principle, completely impossible to obtain information on each of the two pathways, it is still possible to obtain information on the joint effect. However, if one knows, e.g., that pathway x is stimulating and pathway y is inhibiting or that pathway x functions rapidly and pathway y is delayed, then we may be able to extract information about both pathways. Figure 6.7 shows a generic example. In an examination of cerebral autoregulation that we carried out, this strategy was followed. The regulation of the cerebral resistance was believed to consist of three elements: a metabolic part (dependent on local flow), a myogenic part (dependent on local pressure), and a neural part (dependent on acetylcholine, which depends on the pressure at the baroreceptors). After the examination, it turned out that the joint effect could only be achieved if all three

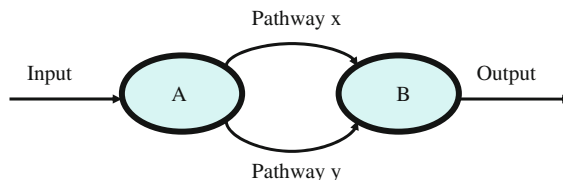


Fig. 6.7 Illustration of a generic problem of many physiologically systems: the effect of one subsystem on another subsystem comprises multiple effects operating through different pathways. Subsystem A influences subsystem B along two different pathways, denoted x and y. If the input to subsystem A is controlled and the output from subsystem B is measured, then it is in principle completely impossible to obtain information on each of the two pathways, but it is still possible to obtain information on the joint effect. However, if one knows, e.g., that pathway x is stimulating and pathway y is inhibiting or that pathway x functions rapidly and pathway y is delayed, then we may be able to extract information about both pathways.

parts were active and that they have to be present in a certain combination to provide the correct time course for the joint effect.

The case studies show that mathematical modelling in medicine is highly important. We have argued that mechanisms-based models can be used for extracting measurements in relation to experiments and that non-trivial measurements cannot be made without using models. Thus, models and experiments are two sides of the same coin. One cannot have one without the other. Furthermore, we have introduced the new term the “mathematical microscope” as a method for using mathematics in accessing information about reality that is otherwise inaccessible. Another conclusion is that, in reality, models are often used in a detective-like way to investigate the consequences of different hypotheses. One can play with a model and as a result investigate it. This is a strategy that cannot be used in (non-mathematical) clinical treatment and research. By clarifying connections and relations, models help reveal mechanisms and develop theories. In the sciences and medicine, a link is often missing in the chain of a system which can be made visible with the aid of the mathematical microscope. The mathematical microscope can be used to obtain a “fingerprint” of the system in question. Finally, we would like to emphasize that the mathematical microscope serves not only as a lens that clarifies a blurry picture, but also as a tool that unveils profound truths. The case studies for type 1 and type 2 diabetes, depression, cardiovascular diseases, and the combination of these along with their interactions, the grey triangle in the metabolic syndrome, are given. They show the delicate and various roles of mathematical models as well as the benefits of mathematical modelling in medicine.

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Part II

Imaging and Sensors

Chapter 7

Magnetic Resonance Imaging of Pancreatic β -Cells

Patrick F. Antkowiak, Raghavendra G. Mirmira, and Frederick H. Epstein

Water, water, everywhere

Samuel Coleridge, *The Rime of the Ancient Mariner*

Abstract The destruction and dysfunction of pancreatic β -cells are at the root of diabetes mellitus. Magnetic resonance imaging (MRI), a non-invasive imaging modality, may play an important role in assessing transplanted islets as well as β -cell mass and function. In addition to conventional MRI of anatomical structure and function, recent advances have led to the development and application of MRI for targeted molecular and cellular imaging. Pancreatic islets incubated *ex vivo* with superparamagnetic iron oxide particles can be visualized as signal hypointensity on MR images, which allows monitoring of viable transplanted islets. β -cells labelled *in vivo* with Mn^{2+} ions can be measured as signal hyperintensity on MR images, since Mn^{2+} ions enter β -cells through voltage-gated Ca^{2+} channels. Compartmental models that describe Mn^{2+} distribution in the pancreas may provide quantitative measurements of β -cell mass and function.

Keywords Magnetic resonance imaging · Pancreatic β -cell · Islet · Contrast agent

7.1 Introduction

Magnetic resonance imaging (MRI) is a powerful technique capable of providing detailed images of structures within the body as well as information about the function of those structures. MRI was invented in the 1970s and early 1980s from the technique called nuclear magnetic resonance (NMR), which had been employed for decades to determine the structure and chemical composition of a

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variety of molecules and systems. In contrast to X-ray computed tomography and nuclear imaging techniques, MRI uses non-ionizing radiation. The field of MRI has expanded significantly since its inception, and new imaging techniques and imaging targets continue to be developed. Particularly, *molecular and cellular MRI*, the visualization of specific molecules and cells in vivo, has recently been an area of intense study and remarkable growth. These techniques have been employed to image many different cell types related to a variety of disease processes. MRI is a potentially well-suited method for imaging pancreatic β -cells due to its high spatial resolution and broad spectrum of contrast-generating mechanisms available; nonetheless, the field of β -cell MRI in the context of diabetes is currently in a state of relative infancy. In this chapter, some current efforts using MRI to image β -cells will be detailed.

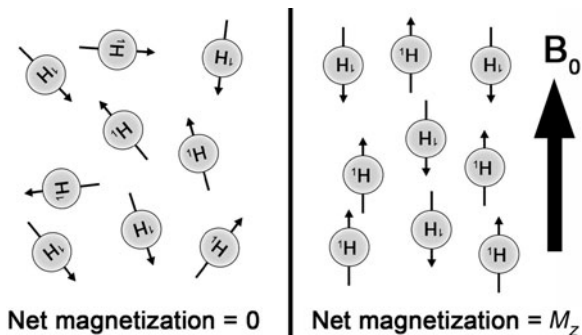
The chapter begins with a discussion of the general physics involved in MRI. After covering the foundation of the MRI signal and some mechanisms for generating signal contrast, the chapter will focus on two applications of β -cell MRI. In the first application, MRI is used to detect transplanted pancreatic islets labelled with a contrast agent. In the second application, a technique called manganese-enhanced MRI is employed to detect pancreatic β -cell function and mass. At the end of this chapter, the reader should have a basic understanding of some of underlying concepts of MRI and how MRI can be applied to image β -cells.

7.2 The Physics of MRI

7.2.1 Nuclear Spin

Only certain atoms with a fundamental property called *nuclear spin* are suitable for detection by MRI. Atoms with either an odd number of protons or an odd number of neutrons possess nuclear spin, which can be positive or negative and exist in multiples of $\frac{1}{2}$. For example, ^1H with one proton and ^{31}P with 15 protons and 16 neutrons both possess non-zero nuclear spin, while ^{16}O with 8 protons and 8 neutrons has zero nuclear spin. This chapter will focus primarily on water protons (^1H), as they are by far the most commonly imaged in MRI due to their high natural abundance in the body. Nuclei with nuclear spin by definition also have a *magnetic moment*, which is conceptually similar to a bar magnet with north and south poles. In the absence of an applied magnetic field, the magnetic moments of nuclei with nuclear spin are randomly oriented. When placed in an externally applied magnetic field, called the B_0 field in MRI, the magnetic moments of nuclei with nuclear spin will tend to align with the external field. However, this alignment with the external magnetic field is not complete, and the spin's magnetic moment forms an angle with the applied magnetic field. Applying the B_0 magnetic field additionally splits the nuclear spins into two quantum energy states: a lower energy state aligned parallel

Fig. 7.1 In the absence of an external magnetic field, nuclear spins (^1H atoms here) are randomly oriented, with net magnetization summing to zero. In the presence of the external magnetic field B_0 , nuclear spins align with or against the field. A slight excess align with the field, creating the net magnetization M_z .



to the external magnetic field (called *spin up*) and a higher energy aligned antiparallel to the external field (called *spin down*), shown in Fig. 7.1. The energy difference between the two states, ΔE , increases linearly with the applied magnetic field. The Boltzmann equation below describes the ratio of the spin-down nuclei n_{down} to the spin-up nuclei n_{up} :

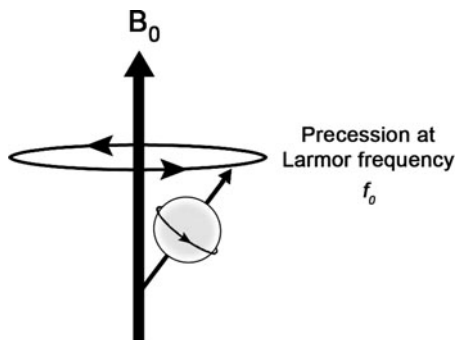
$$\frac{n_{\text{down}}}{n_{\text{up}}} = \exp[-\Delta E/(k_B T)] \quad (7.1)$$

where k_B is Boltzmann's constant and T is the absolute temperature in Kelvin. For the case of ^1H water protons at body temperature in a 1.5 Tesla magnetic field, the ratio of spin-up to spin-down nuclei is approximately 1.000099. There is a slight preference for spins to occupy the lower energy state – if we consider one million water protons, approximately five more will be in the lower energy state than in the higher energy state. Thus, MRI can be considered a fundamentally insensitive imaging technique, since its signal is derived from only a few spins per million. The net excess of protons aligned with the B_0 field supplies the net *longitudinal magnetization* M_z and represents a key contribution to the observed MR signal. Transitions between the energy states can be induced using an external radiofrequency (RF) electromagnetic field or through fluctuations in the local magnetic field, discussed later in the chapter.

7.2.2 The Larmor Frequency

The magnetic moments *precess*, or rotate, around the axis of the B_0 magnetic field at a frequency called the *Larmor frequency* f_0 , as shown in Fig. 7.2. This behaviour is analogous to a spinning top or gyroscope precessing about its axis under the field of gravity. The Larmor frequency is proportional to the magnitude of the applied magnetic field. The *gyromagnetic ratio* γ , unique to a given nucleus, is the proportionality constant that relates the Larmor precession frequency to the magnitude of

Fig. 7.2 Nuclear spins precess about the external magnetic field B_0 at the Larmor frequency f_0



the applied magnetic field. Typically, units for $\gamma/2\pi$ are given in megahertz (MHz) per Tesla (T). For example, $\gamma/2\pi$ for ^1H is 42.58 MHz/T. The Larmor frequency can be calculated given the magnitude of the B_0 field and the gyromagnetic ratio using the following equation:

$$f_0 = \frac{\gamma}{2\pi} B_0 \quad (7.2)$$

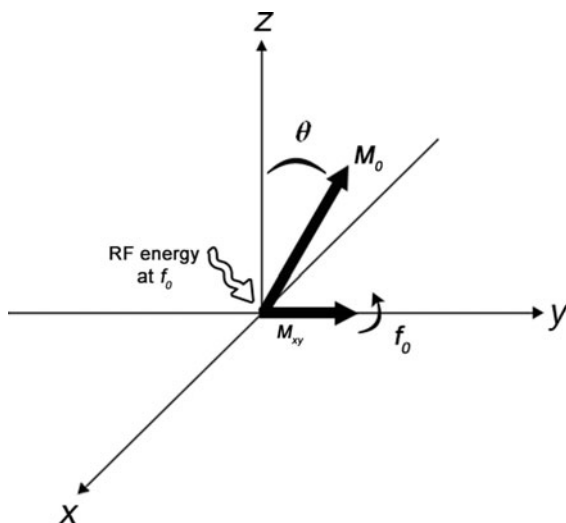
In the common case of clinical imaging using ^1H water protons at 1.5 T, the Larmor frequency is approximately 64 MHz. That is, the magnetic moment of a water proton precesses about the applied magnetic field 64 million times in 1 s. The ΔE in (7.1) is directly proportional to f_0 , so there is a tendency to image nuclei with high γ and to image at high field strengths to maximize the imaging signal. Most clinical MR scanners have a B_0 field strength between 0.1 and 3.0 T, although some research magnets have field strengths up to 9.4 T. Animal research magnets generally have higher magnetic field strengths, around 7.0–11.7 T.

7.2.3 Radiofrequency Excitation and the Free Induction Decay

Changes in the spin energy states of magnetically aligned nuclei can be induced by applying radiofrequency electromagnetic pulses (*RF pulses*) using a radiofrequency coil (*RF coil*) at the Larmor frequency. Energy applied at this frequency, and only this frequency, will be absorbed by the nuclear spins, causing them to gain energy and enter an excited state. This phenomenon is known as *resonance*. Eventually, the spins return back to their equilibrium states.

Application of RF energy at the resonance frequency causes the longitudinal magnetization M_z to tip away from the direction of the main B_0 field into the *transverse plane*, creating transverse magnetization called M_{xy} , depicted in Fig. 7.3. This transverse magnetization is ultimately detected by RF receiver coil (often the same coil used to transmit the radiofrequency energy) where it is recorded as the imaging signal. The amount of magnetization tipped away from the direction of the B_0

Fig. 7.3 A pulse of radiofrequency energy applied at the Larmor frequency f_0 tips longitudinal magnetization away from the z -axis by the flip angle θ creating transverse magnetization M_{xy} . The transverse magnetization continues to precess at the Larmor frequency.

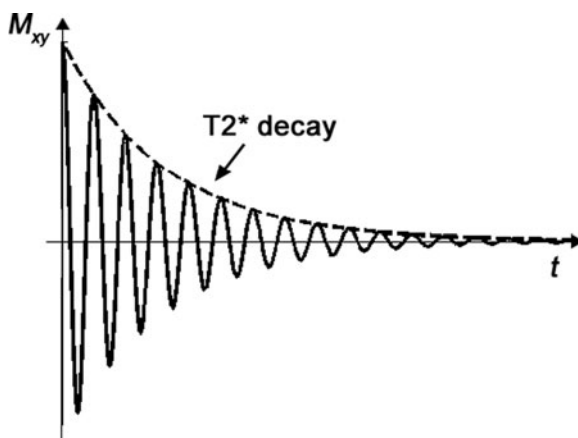


field is determined by the power of the RF pulse and the associated *flip angle* θ . For example, a flip angle of 90° rotates all the longitudinal magnetization into the transverse plane, and a 30° flip angle rotates some magnetization into the transverse plane while still leaving some longitudinal magnetization. A 180° degree flip angle inverts the longitudinal magnetization so that it is aligned against the B_0 field. Applying an RF pulse off-resonance (not at the Larmor frequency) does not cause a transition between spin energy states, and therefore does not tip the longitudinal magnetization away from the direction of the B_0 field, creating no signal.

The transverse magnetization induces a current in the receiver coil due to Faraday's law of induction, which states that a change in magnetic flux induces a current in a closed circuit. The receiver coil is oriented in such a way that it is sensitive only to magnetization in the transverse plane. As transverse magnetization precesses at the Larmor frequency around the B_0 field, the magnetic flux seen by the receiver coil changes, inducing an oscillating current in the receiver coil. The magnitude of this current, the observed *magnetic resonance signal* or *MR signal*, is proportional to the magnitude of the magnetization precessing in the transverse plane. This current, called a *free induction decay (FID)*, can persist for tens of milliseconds. The actual duration of the FID is determined by factors including the homogeneity of the B_0 field and interaction of spins with other nearby spins. The FID is filtered and digitized using an analog-to-digital converter (ADC). The digitized signal is further processed to create an image, but a more complete description of this is beyond the scope of this chapter.

Once magnetization is tipped into the transverse plane, the magnitude of the transverse magnetization begins to decrease exponentially. The exponential decrease of the FID's magnitude, shown in Fig. 7.4, is due to a process called *T_2^* decay* ("tee two star decay"). The time constant T_2^* represents the time it takes for

Fig. 7.4 A free induction decay (FID, *solid line*) is induced in an RF coil by magnetization precessing in the transverse plane. The FID decays exponentially through time due to T_2^* relaxation (*dashed line*).

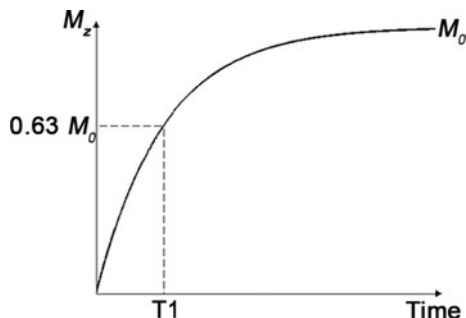


transverse magnetization to decay to $1/e = 37\%$ of its initial value. Of the three major time constants used to describe the behaviour of spin magnetization, T_2^* is always the shortest, and values for T_2^* in vivo range on the order of milliseconds to a few tens of milliseconds. Magnetization in the transverse plane decays due to inhomogeneities or fluctuations in the local magnetic field. These inhomogeneities primarily arise from two sources: (1) intrinsic factors such as interactions with nearby spins (spin–spin interactions) and the surrounding lattice structure, and (2) extrinsic factors including inhomogeneity in the main B_0 field due to imperfect hardware or susceptibility differences in the interface between two different tissues. The T_2^* decay due to extrinsic factors can be eliminated using certain imaging techniques, but the contributions from intrinsic spin–spin interactions cannot be eliminated. Many imaging strategies try to minimize the effects of T_2^* decay, which inherently decreases the amplitude of the received signal and the image signal-to-noise ratio as a consequence.

7.2.4 T_1 Relaxation

The process by which nuclear spins align with an external magnetic field to an equilibrium is called T_1 relaxation. The time constant describing this exponential process is called the T_1 relaxation time, the *spin-lattice relaxation time*, or just simply T_1 . The time T_1 represents the amount of time that it takes longitudinal magnetization to recover to 63% of its equilibrium value, starting from zero longitudinal magnetization, as depicted in Fig. 7.5. Excited spins interact with and transfer energy to spins in the surrounding lattice or tissue, causing the excited spins to lose energy and return to their lower energy equilibrium state aligned with the external magnetic field. T_1 relaxation requires the exchange of energy, and it occurs when the spin experiences another magnetic field fluctuating near the Larmor frequency. This

Fig. 7.5 Longitudinal magnetization M_z recovers due to T_1 relaxation. At time T_1 , the longitudinal magnetization has recovered from zero to 63% of its equilibrium magnetization M_0 .



fluctuating magnetic field is usually due to another proton or atoms with unpaired electrons, as with contrast agents such as gadolinium. For a proton or electron to experience a fluctuating magnetic field, the molecule on which it resides must be moving or tumbling; otherwise, the local magnetic field would be static. In order for the energy transfer between the nuclear spin and the proton or electron to be efficient, the molecule on which the proton or electron reside must be tumbling near the Larmor frequency. Pure water molecules are quite small and tumble too quickly for effective energy transfer, and as a result, the T_1 of pure water is long ($\sim 2\text{--}3$ s). Most water in the body exists in some type of partially bound state, and the T_1 s of many body tissues are on the order of several hundreds of milliseconds. In general, the T_1 s of different tissues in the body are not identical due to their various molecular and cellular compositions. Image acquisition can be manipulated so that the received MR signal is sensitive to differences in T_1 , providing contrast between different types of tissues or tissues in different disease conditions. *T_1 -weighted* image acquisition can make tissues with short T_1 s appear bright and tissues with a longer T_1 appear darker. Additionally, *contrast agents* can be administered to magnify the difference in the image intensity between normal tissue and pathology.

7.2.5 T_2 Relaxation

Longitudinal magnetization flipped by an RF pulse into the transverse plane precesses about the main B_0 field at the Larmor frequency. However, small variations in the local magnetic field causes some magnetization to precess at a slightly lower or higher frequency than the Larmor frequency, resulting in some magnetization gaining or losing *phase* relative to reference magnetization precessing at the Larmor frequency. This process is depicted in Fig. 7.6. This loss of *phase coherence* decreases the magnitude of the received MR signal. The transverse relaxation time, known as the *spin-spin relaxation time*, the *T_2 relaxation time*, or simply *T_2* , is a measure of this loss of phase coherence, and it characterizes the time for the transverse magnetization to decay. Specifically, the time T_2 is the time it takes for

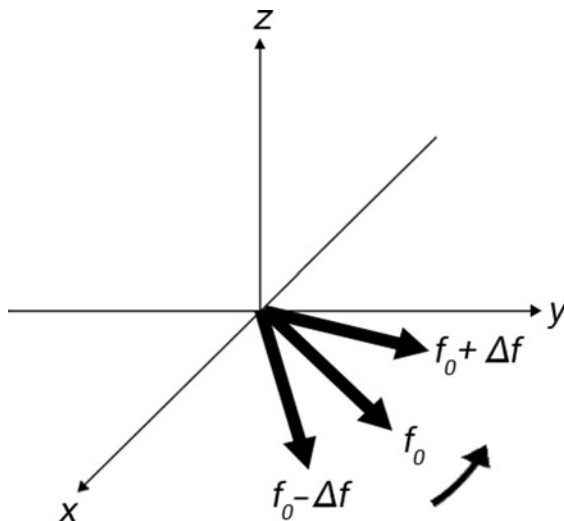


Fig. 7.6 Due to small variations in the main magnetic field, magnetic susceptibility, and spin–spin interactions, there is variation in the precession frequency of the magnetization. Some transverse magnetization precesses exactly at the centre frequency f_0 , some magnetization precesses at faster frequencies such as $f_0 + \Delta f$, and some at slower frequencies such as $f_0 - \Delta f$. The net result is a loss of phase coherence for the transverse magnetization, and an exponentially decreasing signal that occurs more rapidly than T_1 relaxation.

transverse magnetization to decay to 37% of its initial value. T_2 relaxation occurs with or without energy exchange.

As stated before, fluctuations in the magnetic field occur from extrinsic processes (such as imperfect hardware) or intrinsic processes (such as with the interaction of two nearby spins). T_2 relaxation is the result of intrinsic processes, and it cannot be avoided. The equation relating T_2 and T_2^* decay is as follows:

$$\frac{1}{T_2^*} = \frac{1}{T_2} + \frac{1}{T_2'} \quad (7.3)$$

where T_2' is due to the effect of the extrinsic field inhomogeneities. As a consequence of Eq. (7.3), T_2 is always greater than or equal to T_2^* , and among the three main relaxation times in MRI, the following inequality always holds true: $T_1 \geq T_2 \geq T_2^*$. Generally, T_2 in tissue is 5–10 times shorter than T_1 , and many tissue T_2 s are on the order of tens of milliseconds to hundreds of milliseconds.

Spin–spin interactions are dependent on the proximity of adjacent spins and the rates of molecular tumbling. In pure water, ^1H molecules are further apart than in a semi-solid tissue and the amount of spin–spin interaction is lessened. Because of this, pure water has a long T_2 . In large macromolecules with slow molecular tumbling rates, spin–spin interactions are quite efficient and T_2 values may be in the range of a few microseconds to milliseconds. T_2 relaxation time generally varies

with tissue and can be as long as 1 s for cerebrospinal fluid or as short as 10–20 ms for fatty tissues. Image acquisition strategies that exploit the differences in tissue T_2 as a basis for image contrast are known as *T2-weighted* imaging techniques. Similar to T_1 relaxation, T_2 relaxation can be altered with the introduction of T_2 -shortening contrast agents.

7.2.6 Contrast Agents

Contrast, or differences in signal intensity, in MR images can largely be classified into three main categories: (1) proton density-weighted imaging, in which tissue contrast is due to regional differences in the concentration of water protons, (2) *T1-weighted* imaging, in which tissue contrast is due to regional differences in T_1 relaxation times, and (3) *T2-weighted* imaging, in which tissue contrast is due to regional differences in T_2 relaxation times. These contrasts are generated by altering the various parameters governing image acquisition, and quite often these methods alone can generate adequate tissue contrast. However, many imaging studies also utilize *contrast agents*, exogenous materials that can alter the MR signal, to increase the contrast between normal tissues or structures and pathology.

While there are several classes of contrast agents currently used in MRI, this chapter will focus on contrast agents that alter the T_1 and T_2 relaxation times of water molecules in the vicinity of the contrast agent. While all contrast agents affect T_1 , T_2 , and T_2^* , it is convenient to separate contrast agents into two broad categories: contrast agents that primarily shorten T_1 relaxation, and ones that primarily shorten T_2 relaxation, known as *T1 agents* and *T2 agents*, respectively. T_1 agents shorten the tissue T_1 relaxation time comparatively more than they shorten the T_2 relaxation time (which is usually around an order of magnitude smaller than T_1 to begin with), and T_1 agents generally lead to regions of increased signal intensity on T_1 -weighted images. Because T_1 agents increase signal intensity, they are known as positive contrast agents. T_2 agents shorten the tissue T_2 and show up as regions of decreased signal intensity on T_2 -weighted images and are therefore called negative contrast agents. Figure 7.7 compares the effects of T_1 and T_2 agents on relaxation. Examples of T_1 agents are manganese- and gadolinium-based contrast agents, while superparamagnetic iron oxides are an example of T_2 agents.

T_1 and T_2 agents can be characterized by a constant called the *relaxivity*. The relaxivity of a contrast agent relates the concentration of the contrast agent to the observed tissue T_1 or T_2 in the following manner:

$$\frac{1}{T_1} = \frac{1}{T_{10}} + r_1[\text{CA}] \quad (7.4)$$

$$\frac{1}{T_2} = \frac{1}{T_{20}} + r_2[\text{CA}] \quad (7.5)$$

where (7.4) is for a T_1 agent and (7.5) is for a T_2 agent. In those equations, T_1 and T_2 are the observed T_1 and T_2 relaxation times with a tissue contrast agent

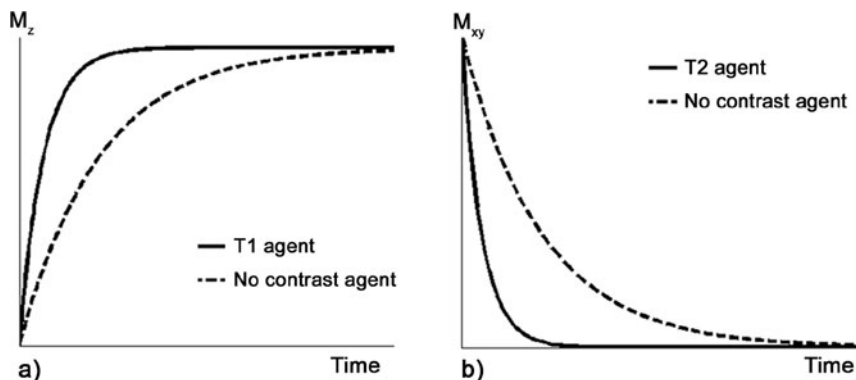


Fig. 7.7 The presence of a contrast agent enhances (a) T_1 relaxation or (b) T_2 relaxation. Relative to the signal intensity without the contrast agent (dashed lines), T_1 shortening (solid line) yields higher signal intensity on T_1 -weighted images, and T_2 shortening (solid line) yields lower signal intensity on T_2 -weighted images.

concentration [CA]. T_{10} and T_{20} are the respective tissue T_1 and T_2 relaxation times in the absence of any contrast agent. The contrast agents' relaxivities, denoted by r_1 and r_2 , have units of $1/(\text{mM s})$ or $\text{mM}^{-1} \text{s}^{-1}$. A contrast agent's relaxivity varies with the main B_0 field strength. With contrast agents it is often convenient to refer to relaxation rates, which are simply $1/T_1$ or $1/T_2$. Generally speaking, contrast agents with larger relaxivities have a greater effect on the tissue relaxation rate. However, this is not necessarily always the case, since contrast agent behaviour in vivo is rather complex. Regardless, comparing the relaxivities of two contrast agents can be a means of ranking their relative effectiveness.

7.2.6.1 T_1 -Shortening Contrast Agents

Most T_1 agents are paramagnetic *gadolinium* (Gd)-based or *manganese* (Mn)-based complexes. These nuclei have high nuclear spin values ($7/2$ for Gd and $5/2$ for Mn) and many unpaired electrons. Both Gd and Mn affect T_1 relaxation through essentially the same dipole–dipole mechanism: They act as small locally fluctuating magnets that interact with nearby water protons. For this to be an efficient mechanism, the local magnetic field due to the contrast agent must be fluctuating at a frequency close to the Larmor frequency.

In its elemental form, gadolinium is fairly toxic. Therefore, all approved Gd-based contrast agents in the United States and Europe contain chelated, or chemically caged, Gd. Specifically, these compounds generally feature an eight-coordinate ligand binding to a Gd molecule, which itself has a coordination site for water binding. The encapsulated Gd atom is quite stable in its chemical shell and is excreted intact from the body. Examples of Gd-based contrast agents are Gd-DTPA (Magnevist[®]) and Gd-DTPA-BMA (Omniscan[®]) among others. Gd-based contrast agents are the most commonly used MR contrast agents and are applicable in the vasculature, myocardium, and brain among other organs. It is important

to note that Gd-based contrast agents have been recently linked to a disease called nephrogenic systemic fibrosis (NSF) in patients with moderate to severe renal failure [8, 30]. NSF is characterized by thickened skin (particularly around the joints), skin lesions, pain in the affected regions, and potential fibrosis of the internal organs [7]. However, NSF is rare and no cases of NSF have been reported in patients with normal kidney function.

Manganese also exhibits toxicity in its elemental form. The clinically approved Mn-based contrast agent Mn-DPDP (Teslascan[®]) operates in a slightly different way than Gd-based agents. A fodipir (DPDP) chelate surrounds a molecule of Mn, which does not have a coordinating site for water to bind and interact with the Mn atom. Instead, Mn slowly dissociates from the surrounding fodipir chemical structure in vivo through transmetallation, primarily with zinc ions. Dissociated Mn ions, which are taken up by cells in the liver, pancreas, and other organs, can interact with water molecules and shorten the T_1 relaxation time through a dipole–dipole interaction. In many animal studies, however, manganese chloride (MnCl_2) is often substituted for Mn-DPDP. Mn-based contrast agents have been used to probe calcium ion channel activity [20, 14, 29].

The relaxivities of many Gd-based and Mn-based contrast agents are fairly similar, and there are two main pathways by which these contrast agents enhance relaxation. The first pathway is known as inner sphere relaxation. This relaxation occurs due to water molecules directly bound to the Gd or Mn metal ion. Inner sphere relaxation is quite efficient, and it is proportional to the number of water molecules coordinated with the metal ion. Increasing the number of coordinated water molecules, however, may decrease the overall stability of the chelate. The effect of inner sphere relaxation is also dependent on the frequency that bulk water chemically exchanges with bound water at the metal's coordination site. The second relaxation pathway is known as outer sphere relaxation. Outer sphere relaxation collectively includes the relaxation of water molecules that hydrate the chelation complex as well as bulk water diffusing around the complex. The relative contributions of inner sphere and outer sphere relaxation vary from contrast agent to contrast agent and can be changed by manipulating the contrast agent's chemistry.

7.2.6.2 T_2 -Shortening Contrast Agents

The chemistry and action of T_2 agents differs from that of T_1 agents. T_2 agents are generally nanometre-sized *superparamagnetic iron oxide nanoparticles* and appear as regions of signal hypointensity on T_2 -weighted or T_2^* -weighted images. They are composed of a core of one or more crystals of maghemite or magnetite (Fe_2O_3 and Fe_3O_4 , respectively), often stabilized by an inert coating such as dextran or a dextran derivative. These iron oxide agents are classified according to particle size, with ultrasmall particles of iron oxide (USPIOs) having a single iron oxide crystal core and a total particle diameter <50 nm, while small particles of iron oxide (SPIOs) consist of multiple iron oxides at the core and have a total particle diameter >50 nm. The particles are superparamagnetic: They have magnetism that exists only

when their spins are aligned in an external magnetic field. Outside of an applied magnetic field, superparamagnetic particles do not exhibit magnetism.

Superparamagnetic iron oxide nanoparticles do not have a coordination site for water, so there is no inner sphere relaxation. Outer sphere relaxation occurs, though its mechanism is slightly different from that of paramagnetic $T1$ agents. Iron oxide nanoparticles are primarily used for their *susceptibility* effects, which increase $T2$ or $T2^*$ relaxivity. A magnetized superparamagnetic iron oxide particle creates a spatially varying magnetic field many times larger than the particle itself. Consequently, spins randomly diffusing nearby lose phase coherence, causing enhanced $T2$ and $T2^*$ relaxation. This magnetic susceptibility phenomenon extends much further than outer sphere relaxation effects, and it is the dominant pathway by which $T2$ agents act. For further discussion on nanoparticles, please refer to [Chapter 9](#) of this publication.

The two main methods of introducing iron oxide particles into tissue are (1) by systemically injecting iron oxide particles in solution or (2) by incubating cells or tissue in media that contain iron oxide particles in vitro. Systemically injected iron oxide nanoparticles are taken up by phagocytic cells, such as circulating monocytes or macrophages in the liver or spleen. Non-phagocytic cells incubated with iron oxide nanoparticles in vitro likely internalize the particles through pinocytosis or other pathways. These labelled cells can then be monitored with $T2$ - or $T2^*$ -weighted imaging. Particle size and modifications to the particle's surface chemistry influence the biodistribution of these agents in vivo.

7.2.6.3 Contrast Agent Compartmentalization and the Effects of Water Exchange

An important, if subtle, nuance separates MR contrast agents from those used in other modalities such as X-ray, SPECT, and PET. Generally, MR contrast agents are not themselves directly detected per se. Instead, the contrast agent's effect on the relaxation times of nearby water protons is detected. Imaging techniques sensitive to these differences in relaxation times can then be employed to translate these differences in relaxation times to image contrast.

Water must diffuse into the small hydration sphere (comprised of the inner and outer spheres) of a $T1$ agent to affect relaxation, and similarly water must be within the reach of a $T2$ agent's magnetic field gradient to cause loss of phase coherence and enhanced relaxation. Because of this, biological membranes and barriers, which limit the distribution volume of the contrast agent itself as well as water diffusion between biological compartments, significantly affect the action of contrast agents in MRI [31]. For simplicity, the remainder of this section will focus on $T1$ agents, although the same concepts hold true for $T2$ agents. The distribution volume of Gd-DTPA, for example, is limited to the intravascular and interstitial spaces, and it cannot typically enter the intercellular space. Normally, the rate of water diffusion within a biological compartment is rapid relative to its $T1$, and contrast agents uniformly shorten the $T1$ of all water in the compartment equally. At the scale of MRI resolution, tissue volumes are heterogeneous and consist of multiple distinct

compartments. The degree that the contrast agent affects water protons in the other compartments depends on the *water exchange* between compartments.

The Bloch-McConnell equations [36] govern the behaviour of a system with compartmentalized $T1$ relaxation rates and water exchanging between the compartments. To gain insight into how water exchange affects the $T1$ relaxation of a composite volume of tissue and the observed MR signal, we will consider the extreme cases of water exchange regimes. If the water exchange rate is very fast relative to the $T1$ s of the tissue compartments, the system is said to be in *fast exchange*. In fast exchange, the system relaxes with a *monoexponential* $T1$ that is the weighted average of the size of the compartments multiplied by their individual $T1$ relaxation rates as follows:

$$\frac{1}{T1} = f_a \frac{1}{T1_a} + f_b \frac{1}{T1_b} \quad (7.6)$$

where f_a and f_b are the relative sizes of the compartments in the composite volume of tissue, and $T1_a$ and $T1_b$ are the individual $T1$ relaxation times of each respective compartment.

If the water exchange rate between compartments is very slow relative to their respective $T1$ relaxation rates, then the system is said to be in *slow exchange*. In this case, the compartments relax with essentially two uncoupled $T1$ relaxation rates. This system will exhibit *biexponential* $T1$ relaxation, and the observed $T1$ relaxation curve will be the sum of the discrete monoexponential $T1$ relaxation curves from each compartment. Figure 7.8 considers a volume of tissue with two compartments of equal sizes. Compartment A has a short $T1$, and compartment B has a longer $T1$. The water exchange rate significantly affects the observed $T1$ relaxation, and consequently the measured signal intensity, in this system. Few biological systems are actually in slow exchange, although high doses of contrast agents can shift a system into the slow exchange regime.

Finally, if the water exchange rate of the system is between fast exchange and slow exchange, the system is said to be in *intermediate exchange*, a case that may occur quite commonly with contrast agents in vivo. A system in intermediate exchange will exhibit biexponential relaxation, but the effective compartment sizes and $T1$ s will be different from the true biological compartment sizes and $T1$ s. Therefore, the effects of water exchange between compartments must be accounted for when describing a system in intermediate water exchange in order to calculate the actual compartment sizes of the system.

Since the water exchange regime is by definition relative to compartmental $T1$ s, addition of compartment-specific contrast agent can shift the water exchange rate from fast to intermediate exchange regimes. For example, the intracellular space may be deliberately made to contain a higher concentration of contrast agent than the other spaces through the cell labelling process. While a two compartment system is generally an oversimplification of the complex structure of biological tissue, two compartment systems have been quite useful for explaining the action of water exchange on contrast agents in several studies [18, 19].

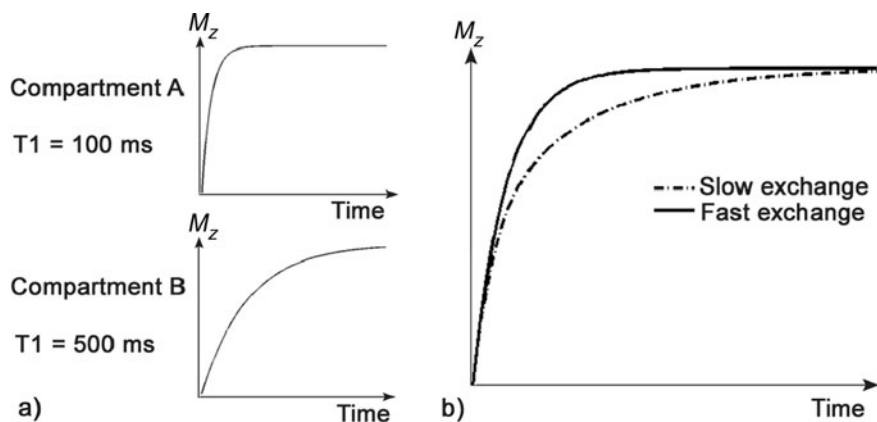


Fig. 7.8 A composite volume of tissue is composed in this example of two equally sized compartments. (a) Compartment A has a short T_1 relaxation time ($T_{1A} = 100$ ms, *top*) and compartment B has a longer relaxation time ($T_{1B} = 500$ ms, *bottom*). (b) Comparison of tissue T_1 relaxation with slow water exchange (dot-dash line) and fast water exchange (solid line). In fast exchange, the longitudinal magnetization of the composite tissue volume returns to equilibrium sooner than in slow exchange. Fast exchange results in greater signal enhancement relative to slow exchange.

7.2.7 Pulse Sequences

MR images are acquired through the application of a sequence of RF pulses, which excite the magnetization, and magnetic field *gradient pulses*, which encode information about the spatial distribution of the magnetization into the MR signal. The specific manner in which RF pulses and gradient pulses are played out is known as a *pulse sequence*. MR image contrast can be manipulated by changing the strength and timing of these pulses. A full treatment of gradient pulses is beyond the scope of this chapter, so the discussion will focus on the timing of the RF pulses and data acquisition periods for some examples of pulse sequences used to acquire T_1 - and T_2 -weighted images.

7.2.7.1 Gradient Echo Pulse Sequence

The most basic pulse sequence is called a *gradient echo sequence*. In a gradient echo pulse sequence, an excitation RF pulse with a flip angle tips longitudinal magnetization away from the direction of the B_0 field into the transverse plane, creating an MR signal that is sampled by an analog-to-digital converter (ADC). The time between the centre of the RF pulse and the peak of the received signal is called the *echo time TE*. This process of exciting the magnetization with an RF pulse and sampling the signal with an ADC is repeated many times, in order to fully acquire the data according to certain image formation and signal processing requirements. The time it takes to repeat the RF pulses is known as the *repetition time TR*. Gradient echo imaging with short TE and short or long TR can be used to create proton density-weighted

or T_1 -weighted images, depending on the flip angle and TR. Gradient echo imaging with a longer TE can be used to create T_2^* -weighted images.

7.2.7.2 Spin Echo Pulse Sequence

A *spin echo* pulse sequence employs an additional refocusing RF pulse to eliminate the loss of phase coherence due to magnetic field inhomogeneities. In a spin echo pulse sequence, an excitation RF pulse with a 90° flip angle tips longitudinal magnetization into the transverse plane, as in a gradient echo pulse sequence. Then, at time TE/2 after the initial RF excitation pulse, a 180° refocusing pulse is applied. Rather than tipping down longitudinal magnetization from the z -axis, this 180° pulse is oriented in such a way as to flip the transverse magnetization about the y -axis. The phase that the transverse magnetization accumulates due to magnetic field inhomogeneity during the subsequent period of TE/2 after the 180° refocusing pulse perfectly cancels out the phase accumulated during the initial TE/2 period. Because of this, spin echo pulse sequences are insensitive to signal loss from inhomogeneity in the main field (T_2' decay). At time TE after the RF excitation pulse, the signal is sampled by an ADC. This process is repeated every TR until the data is sufficiently sampled.

Due to the spin echo's relative insensitivity to signal loss from B_0 inhomogeneity, TE can be widely adjusted to manipulate image contrast. With short TE and short TR, T_1 weighting is achieved. Long TE and long TR yield T_2 weighting, while short TE and long TR give proton density weighting. Signal-to-noise ratio is high in spin echo sequences due to the elimination of T_2' signal loss, though scan time is increased relative to gradient echo pulse sequences due to the additional pulses required in the spin echo pulse sequence.

7.2.7.3 Inversion Recovery Gradient Echo Pulse Sequence

In an *inversion recovery* gradient echo pulse sequence, the longitudinal magnetization is initially inverted with a 180° RF pulse. Subsequently, the magnetization undergoes T_1 relaxation towards equilibrium for a time denoted by the *inversion time* TI. After TI has elapsed, a 90° RF excitation pulse is played to tip the magnetization into the transverse plane, where the data are acquired by the ADC at time TE after the excitation RF pulse in the same manner as in the gradient echo pulse sequence. The process is again repeated every TR until the data are sufficiently sampled.

Inversion recovery pulse sequences typically acquire images with T_1 weighting. In particular, inversion recovery pulse sequences can nullify the signal from specific types of tissue by placing the excitation RF pulse at the *zero crossing time* or the *null time* of the tissue of interest. The null time occurs when the signal from inverted spins crosses the zero point due to T_1 relaxation. The null time can be calculated from T_1 in the following manner:

$$T_{\text{null}} = \ln(2) \times T_1 \quad (7.7)$$

Excellent contrast between two tissues with different T_1 can be generated by using inversion recovery imaging with the inversion time set at the null time of one tissue.

A Look-Locker pulse sequence is a special type of inversion recovery gradient echo imaging pulse sequence that can be used to quantify spatially localized T_1 relaxation. After the initial RF pulse is applied to invert the magnetization, a series of gradient echo images are acquired at an array of inversion times to generate a sequence of images that depicts, pixel by pixel, T_1 relaxation. Look-Locker pulse sequences are used when accurate estimation of T_1 relaxation is desired.

7.3 β -Cell MRI

7.3.1 *Introduction*

Pancreatic β -cells represent a fundamentally challenging imaging target. β -cells reside in the pancreatic islets of Langerhans, small structures diffusely spread within the pancreatic parenchyma. Islets represent approximately 1–2% of the mass of the pancreas, with β -cells composing between 50 and 80% of islet volume [6]. With a mean size ~ 100 – $200\ \mu\text{m}$ [10], islets are too small to be directly visualized on conventional clinical scanners whose image resolutions are in the millimetre range. High-field animal imaging systems could achieve the resolution ($<100\ \mu\text{m}$) necessary to view islets within the pancreatic tissue; however, the intrinsic contrast between islets and the surrounding tissue is likely minimal. Additionally, the pancreas moves with respiration, which must be accounted for either through breath-holding, respiratory gating, or imaging schemes that account for respiratory motion. Given that it is a difficult task, the question remains: Why do we want to image β -cells?

Currently, there is no established method for non-invasively monitoring many of the key cellular events such as loss of β -cell mass and function that occur in the disease process of diabetes mellitus. By developing methods to assess β -cell mass and function, it may be possible to better monitor potential new therapies for diabetes and to gain insight into the underlying events that occur in the diabetic disease process.

7.3.2 *MRI of Islet Transplantation*

One recent potential treatment for type 1 diabetes involves transplanting purified donor islets into the liver [28]. While many patients achieve insulin independence after donor islet transplantation, a significant fraction of these transplants fail, and the factors influencing islet failure are not completely understood at this time [27]. Developing ways to visualize these transplants and monitor their fate would aid in

understanding ways to improve transplant outcomes. Please refer to [Chapter 16](#) for more on the current state of islet transplantation in type 1 diabetes.

Jirak and colleagues [15] first reported using MRI to detect transplanted pancreatic islets in rats in 2004. In this pilot study, the investigators incubated isolated rat islets with the clinically approved SPIO contrast agent Resovist[®] for 2–3 days and performed MRI after injecting the labelled islets into the liver. The liver was imaged both in vivo in the intact animal and in vitro after excision. The authors were able to visualize the labelled islets in vivo as focal areas of hypointense signal in the liver using $T2^*$ -weighted gradient echo imaging. They reported that the SPIO label generally persisted for the 22 week duration of the study, although the number of labelled islets diminished over time. They showed that the SPIO-labelled islets had slightly decreased insulin production compared to unlabelled islets, though they were still able to restore normoglycaemia in a rat model of T1DM. This study's important finding is that imaging labelled transplanted islets is feasible with MRI, and several similar studies followed in a variety of animal and transplantation models.

Quantum Mechanics Playing into Macro-space

The postulation of quantized physical quantities, as different from continuous ones, was first introduced into physics in a model to describe the release of electrons from matter after absorption of light, so-called photo-effect (Einstein A (1905) Über einen die Erzeugung und Verwandlung des Lichtes betreffenden heuristischen Gesichtspunkt. *Annalen der Physik* 322(6):132–148). The resulting kinetic energies of the released electrons, moving in an accelerating electric field, could only be explained when quantized energy states were postulated for the initially bound electrons. Also the spectrum of thermal radiation could only be interpreted if it was connected to discontinuous quantized states (Einstein A (1905) Über die von der molekularkinetischen Theorie der Wärme geforderte Bewegung von in ruhenden Flüssigkeiten suspendierten Teilchen. *Annalen der Physik* 322(8):549–560, based on: Planck M (1901) Über das Gesetz der Energieverteilung im Normalspectrum. *Annalen der Physik* 309(3): 553–563).

In a rapid scientific process, quantum mechanics was then developed to supplement classical physics. The term “quantum physics” was first introduced by M. Planck in his book *The Universe in the Light of Modern Physics* (1931, translation of two books *Das Weltbild der neuen Physik* and *Physikalische Gesetzmäßigkeit im Lichte neuerer Forschung*, published by J A Barth, Leipzig 1929). Nowadays, the term “quantum jump” was successfully entered into every day language, and the philosophical views behind it were adopted into the cultural background of recognition.

Technological applications are abundant, as MRI, and they are accepted as a matter of course. No patient will worry about being disintegrated into quantum details, and interpretations taken are extended to their macroscopic relevance, for example, in the distinction between malicious and healthy tissue. Quantum physics thus has emanated from the academic to the socially relevant, providing modern technological tools that could never be developed exclusively on the basis of classical physics.

Added by the editors

Evgenov and colleagues [11, 12] reported comparable results with dual-labelled (near-infrared fluorescent dye for optical imaging and SPIO for MRI) islets transplanted in the kidney capsule and liver in mice. They observed no changes in the T_2 relaxation time of transplanted islets for the duration of the 188-day study, indicating that the transplanted islets were intact and contained the SPIO label. Additionally, they confirmed that kidney capsule islet transplants still contained the label with ex vivo fluorescence imaging. Similar studies using SPIO-labelled transplanted islets were reported at a variety of field strengths, from 1.5 to 7.0 T [4, 21, 22]. A goal in the field is to eventually translate the technique to be used clinically in humans. In a step towards this, Medarova and colleagues [25] have reported success in monitoring SPIO-labelled islets transplanted in the liver and the kidney capsule in a non-human primate (baboon) model. Figure 7.9 shows in vivo MRI of SPIO-labelled pancreatic islets transplanted into the kidney capsule of a baboon. The signal dropout due to the transplanted islets (*white arrow*) is appreciable in this T_2^* -weighted gradient echo image. Recently, Toso and colleagues [33] published a preliminary study featuring SPIO-labelled islets transplanted in the liver in four human patients, which is, to our knowledge, the first such study in human.

While these studies with SPIO-labelled islets have shown effectiveness, several groups are exploring ways to improve the technique. Tai and associates reported that SPIO-labelling efficiency could be increased by using electroporation and by adding poly-L-lysine to the labelling medium, although Kim and colleagues [16, 32] reported that incubating islets with the SPIO agent Resovist[®] alone was sufficient. Barnett and colleagues [3] encapsulated islets inside a thin alginate membrane prepared with the SPIO agent Feridex[®], making the encapsulated islets magnetic resonance-detectable. This alginate membrane is permeable to insulin and metabolites, but not to native antibodies, and so remains isolated from immune system

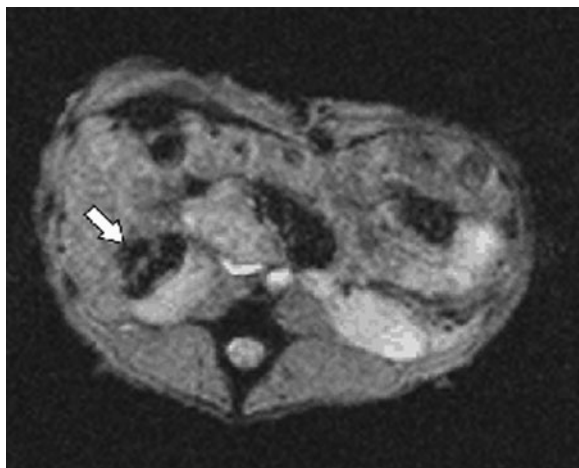


Fig. 7.9 Axial gradient echo image of a baboon abdomen. SPIO-labelled transplanted pancreatic islets (*white arrow*) appear as a large region of hypointense signal beneath the kidney capsule. With kind permission from Anna Moore.

attack. The Feridex-prepared immuno-encapsulated islets were easily distinguishable areas of signal hypointensity in the liver visible in $T2^*$ -weighted images. An important benefit of this technique is that it could reduce or avoid the need for an immunosuppressive drug regimen after transplantation, which is otherwise used to prevent immune destruction of the transplanted islets.

The vast majority of MRI studies of transplanted islets use SPIOs because they are approved for use in humans, they are easily incorporated into islets, and they cause fairly large signal dropout effects. However, Biancone and co-workers [5] labelled islets with the Gd-based agent GdHPDO3A, which produces positive contrast. Much like the SPIO-labelled islets, their Gd-labelled islets maintained viability, verified by gene expression and insulin secretion assays. Using a $T1$ -weighted spin echo pulse sequence, they observed the labelled islets as areas of hyperintense signal in the liver and in the kidney capsule. However, by the end of their 65-day study, the area with Gd-enhanced pixels had significantly decreased, which the authors attributed to islet release of contrast agent. Their rationale for using a positive contrast agent is that positive contrast agents are advantageous for detecting labelled cell grafts in regions endowed with a low intrinsic MR signal. Inversion recovery imaging could potentially increase their sensitivity to detect Gd-labelled islets in future studies.

7.3.3 Manganese-Enhanced MRI of β -Cells

A promising method for probing function and mass of endogenous β -cells is through the use of *manganese-enhanced MRI (MEMRI)*. The $T1$ -shortening contrast agent manganese (Mn^{2+}) has been used as a calcium (Ca^{2+}) analogue in a variety of studies due to the ions' similar size and valence. β -cell insulin release, stimulated by intracellular glucose transport, is immediately preceded by Ca^{2+} -ion influx through voltage-gated Ca^{2+} channels (VGCCs). The reader is invited to refer to [Chapter 2](#) for a more in-depth treatment of insulin exocytosis. A simplification of the β -cell insulin release pathway is shown in [Fig. 7.10](#). Because of the tight coupling between β -cell glucose intake and Ca^{2+} channel activity, MEMRI is being explored as a potential means to probe the function of glucose-stimulated β -cells.

The first results of functional β -cell imaging with MEMRI were presented in a study by Gimi et al. [13]. The authors performed a variety of imaging experiments *in vitro* with isolated β -cells and islets incubated with various levels of glucose and Mn^{2+} to prove that MEMRI could be used as an indicator of β -cell function. They hypothesized that $T1$ -weighted MRI of β -cells and islets stimulated with high concentrations of glucose and Mn^{2+} would show increased signal intensity relative to non-stimulated islets and β -cells due to increased Mn^{2+} internalization through VGCCs. In cultured β -cells and islets incubated with varying concentrations of glucose and a constant concentration of Mn^{2+} , they measured higher signal intensities with increasing glucose concentration using $T1$ -weighted gradient echo and spin echo imaging. Islets incubated with the maximal level of glucose had a

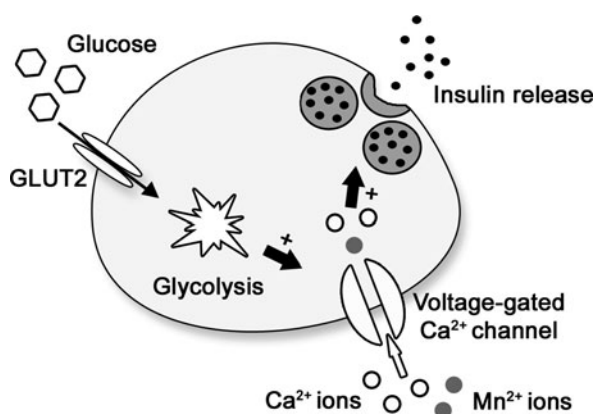


Fig. 7.10 Glucose-stimulated insulin release in the β -cell and applications for Mn^{2+} -enhanced MRI. Glucose enters the β -cell through a GLUT2 transporter, where it undergoes glycolysis. Further down the pathway, this depolarizes the β -cell and opens voltage-gated Ca^{2+} channels. Ca^{2+} ion (small white circles) influx stimulates insulin release. Mn^{2+} (small grey circles) enters the β -cell through voltage-gated Ca^{2+} channels due to similar ion size and valence.

45% signal increase relative to islets incubated without glucose at the same concentration of Mn^{2+} . The authors also measured the intracellular Mn^{2+} concentration after glucose stimulation and found that it increased with Mn^{2+} concentration during incubation. Both of these results indicated that Mn^{2+} influx was dependent upon β -cell glucose stimulation. Remarkably, the authors were able to image single rodent islets using specialized microcoils with in-plane imaging resolutions up to 14 μm , a resolution that is near the water diffusion limit, the fundamentally highest resolution that can be achieved with MRI. Importantly, the authors reported that Mn^{2+} did not significantly hinder physiological β -cell insulin secretion at the relevant Mn^{2+} concentrations, which paved the way for in vivo MEMRI studies.

MEMRI was recently employed to detect functional β -cell mass in vivo in non-diabetic and diabetic mice [1]. Serial MRI was performed in mice after injecting MnCl_2 , either with or without glucose injection to stimulate β -cell Ca^{2+} activity. For imaging, an inversion recovery pulse sequence was used, with the inversion time set to null the pancreas before MnCl_2 injection. After MnCl_2 injection, the $T1$ in the pancreas shortened as Mn^{2+} accumulated both inside glucose-stimulated β -cells and also in other water compartments, and the shortened $T1$ was detected as increased signal intensity on the $T1$ -weighted images shown in Fig. 7.11. The time course of pancreatic Mn^{2+} enhancement was measured by collecting images at approximately 3-min intervals for up to 45 min after MnCl_2 injection, and signal enhancement kinetics were observed as β -cells took up and retained Mn^{2+} . These studies were performed in non-diabetic mice and in a type 1 diabetes mouse model. Type 1 diabetes was induced with injection of streptozotocin (STZ), a drug that is selectively toxic to β -cells. The diabetic mice were divided into two categories: high-dose STZ

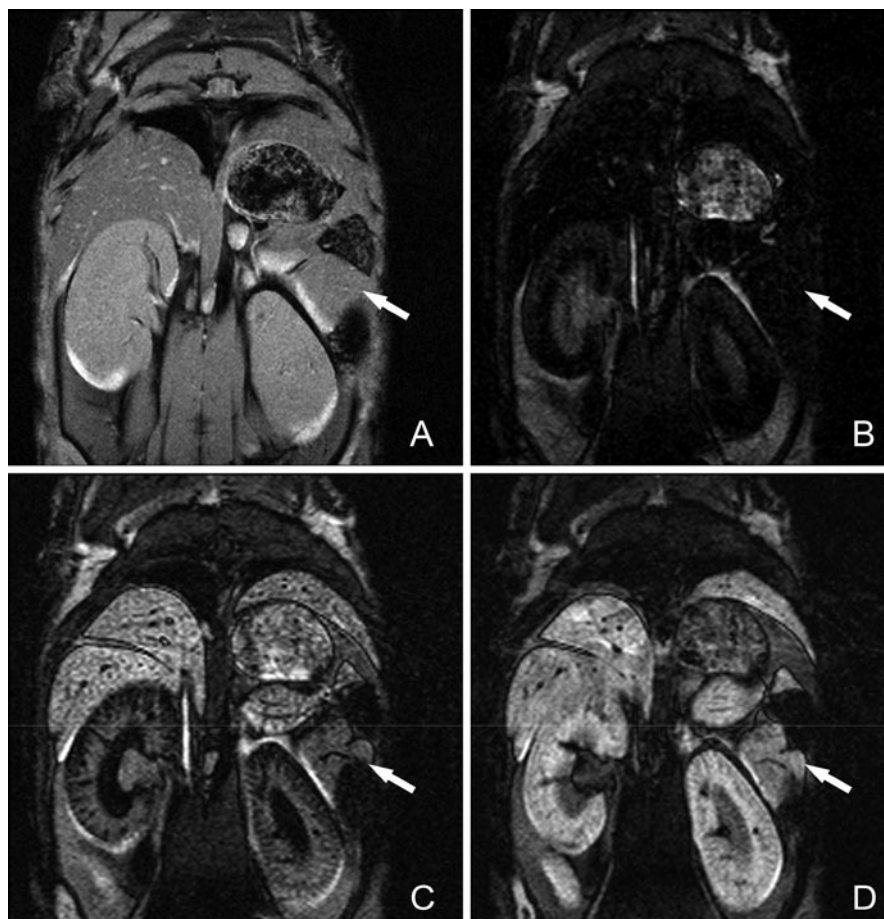


Fig. 7.11 Mn^{2+} -enhanced magnetic resonance (MR) images of mouse pancreas. (a) Gradient echo anatomic reference image. (b) Precontrast inversion recovery image with the pancreas nulled. Inversion recovery images acquired 5 min (c) and 45 min (d) after injection of $MnCl_2$. Signal intensity in the pancreas initially increased rapidly and reached a plateau ~ 15 min after injection of $MnCl_2$. Arrows indicate location of the pancreas. (Reprinted from Antkowiak [1]).

mice with severe diabetes (a single 180 mg/kg injection) and low-dose STZ mice with moderate diabetes (5 consecutive days of 50 mg/kg STZ injection). To compare pancreatic enhancement between groups, normalized pancreatic signal was used (pancreatic signal divided by liver plateau signal), which accounted for variations in the input of Mn^{2+} due to factors such as cardiac output. Figure 7.12 depicts the normalized pancreatic signal vs. time intensity curve for non-diabetic mice, and the normalized pancreatic signal vs. time intensity curves for both groups of diabetic mice are shown in Fig. 7.13. In normal mice, a 51% increase in the normalized signal was observed with glucose stimulation relative to non-stimulation. In the low-dose STZ mice, the increase was only 20% after glucose stimulation. Finally, a 9% signal

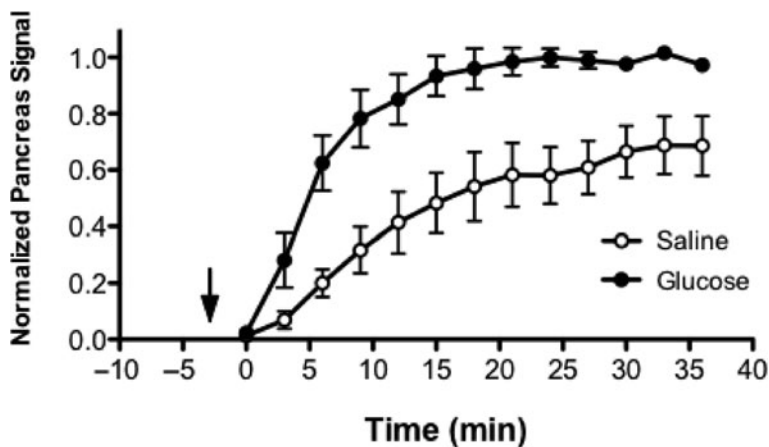


Fig. 7.12 Normalized time-signal intensity curves (TICs) for the pancreas after saline and glucose infusions in normal mice. Mn^{2+} -enhanced MR signal in the pancreas (normalized to liver signal) after saline and glucose infusions is plotted vs. time after MnCl_2 injection. Arrow denotes time of injection for saline/glucose, and MnCl_2 was injected at time = 0 min. Data points represent means $\pm$ SE from five mice. (Reprinted from Antkowiak [1]).

increase was measured with glucose stimulation relative to non-stimulation in high-dose STZ mice. This study showed that MEMRI applied *in vivo* can detect increased β -cell function in normal mice after glucose stimulation, and it also showed sensitivity to changes in functional β -cell mass exhibited in the low- and high-dose STZ mice.

Interestingly, the signal increase measured with glucose stimulation was quite high ($\sim 50\%$) relative to β -cell mass ($\sim 1\text{--}2\%$ of the pancreas). If extra manganese due to glucose stimulation is shortening the T_1 of water only inside β -cells, then how can such a large signal increase occur? While glucose-stimulated perfusion increases may account for a small part of this, it was hypothesized that water exchange between β -cells and the pancreatic parenchyma might better explain this phenomenon. With water exchange, the higher concentration of intra- β -cell Mn^{2+} shortens the T_1 not only of intra- β -cell water, but also of extracellular water. Therefore, a volume of water much larger than just inside β -cells would contribute to the increased signal intensities measured in Fig. 7.12.

Due to the heterogeneous distribution of Mn^{2+} in the pancreas, the intra- β -cell compartment and extra- β -cell compartment each have a distinct T_1 . The intra- β -cell T_1 is likely shorter than the extra- β -cell T_1 due to the preferential uptake of Mn^{2+} by glucose-stimulated β -cells. Each compartment also has a longitudinal magnetization related to the size of the compartment. The relative size of the intra- β -cell compartment, called the intra- β -cell compartment fraction, may be calculated by applying a two compartment model of T_1 relaxation that includes the effects of water exchange between compartments to a T_1 relaxation curve of the Mn^{2+} -enhanced pancreas.

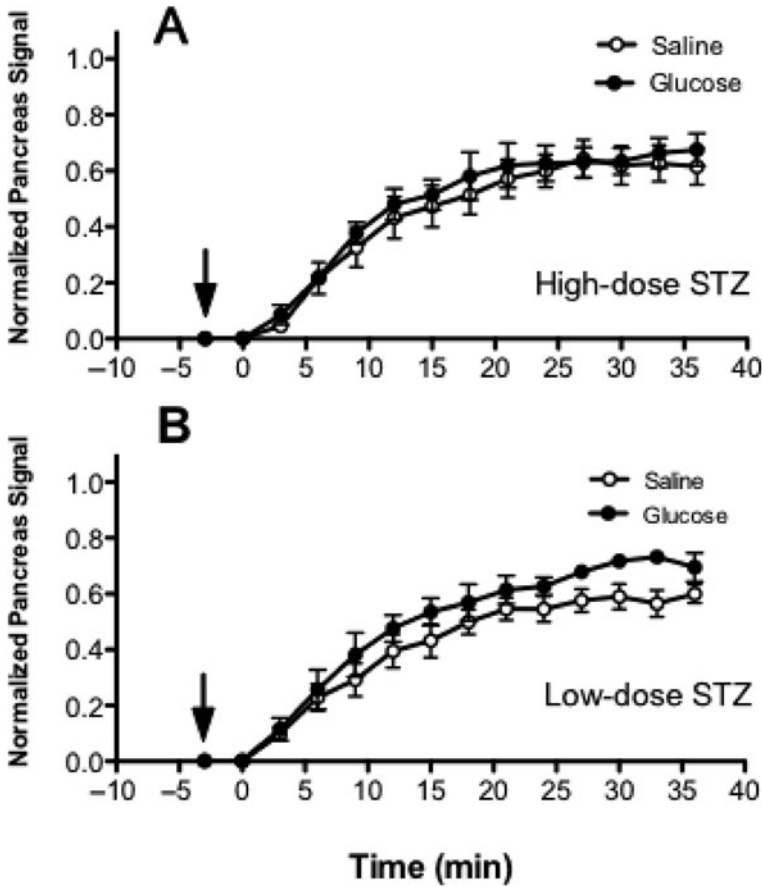


Fig. 7.13 Normalized TICs for the pancreas after saline and glucose infusions in STZ-diabetic mice. Mn^{2+} -enhanced MR signal in the pancreas (normalized to liver signal) after saline and glucose infusions is plotted vs. time after $MnCl_2$ injection. Arrows denote time of injection for saline/glucose, and $MnCl_2$ was injected at time = 0 min. (a) High-dose STZ mice. (b) Low-dose STZ mice. Data points represent means $\pm$ SE of five high-dose STZ mice and four low-dose STZ mice. (Reprinted from Antkowiak [1]).

We hypothesized that the intra- β -cell compartment fraction may represent a surrogate measurement of β -cell mass. To test this hypothesis, Look-Locker MRI was used to measure T_1 relaxation in the pancreas in vivo after glucose + $MnCl_2$ injection, and a two compartment water exchange analysis model was applied to the longitudinal T_1 relaxation recovery curve to calculate the intra- β -cell compartment fraction and intra- β -cell T_1 [2]. Pancreatic T_1 relaxation was imaged in non-diabetic mice and in a high-dose STZ mouse model of diabetes 1 h after glucose + $MnCl_2$ injection. Two compartment water exchange analysis revealed that two compartments of protons, relaxing with different T_1 s, were present in the pancreas. The intra- β -cell T_1 , indicative of β -cell labelling with Mn^{2+} , was significantly shorter in

non-diabetic mice than in diabetic mice. The intra- β -cell fraction in non-diabetic mice was 4.0%, while the intra- β -cell fraction was significantly less (2.5%) in diabetic mice. Since STZ injection greatly reduces β -cell mass, the residual 2.5% intracellular fraction in STZ-treated mice probably represents Mn^{2+} uptake by other excitable cells in the pancreas, such as nerve cells, since the pancreas is known to be innervated [23]. Then, the intracellular fraction difference between the two groups, 1.5%, may signify the β -cell mass of the non-diabetic mice. This method of non-invasively labelling β -cells with Mn^{2+} and performing two compartment water exchange analysis of pancreatic $T1$ relaxation may be a promising way to quantitatively measure β -cell mass and function. However, the validity of the model and the sensitivity of this technique to detect more gradual decreases in β -cell mass remain to be addressed.

7.4 Conclusions

MRI may allow for the development of non-invasive techniques capable of imaging β -cell mass and function, which are desired for both scientific diabetes research and clinical diagnostics. This chapter discussed some fundamentals of the MR signal and presented how those fundamentals may be applied to image β -cells in the clinically relevant applications of islet transplants and Mn^{2+} -enhanced MRI of β -cell function and mass. While this chapter focused on two specific applications of MRI to β -cell imaging, MRI has additionally been employed to observe various facets of islet physiology and the diabetic disease process. Specifically, we note its utility to measure the immune cell infiltration that precipitates T1DM [24, 26] and the accompanying changes in the islet microvasculature [9, 34]. Moreover, MR contrast agents are not limited in scope to compounds that only alter $T1$ or $T2$ relaxation. Certain contrast agents called CEST or PARACEST agents shift the resonant frequency of local water protons and have been used to detect markers of cellular metabolism, including glucose, a property which could be potentially exploited to image β -cells [35, 37]. An added advantage of CEST agents is that they must be activated by an RF pulse at a specific off-resonance frequency, making them a selectively tunable contrast agent. MRI may provide important tools to study β -cells, applicable in basic science and clinical research studies.

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Chapter 8

Mapping the β -Cell in 3D at the Nanoscale Using Novel Cellular Electron Tomography and Computational Approaches

Andrew B. Noske and Brad J. Marsh

Abstract The three-dimensional (3D) clusters of highly specialized cells known as the “islets of Langerhans” (“islets”) which are distributed throughout the pancreas collectively serve as the coordinately regulated tissue that is commonly referred to as the “endocrine pancreas”. In mammals, the so-called β -cells that predominate within each islet are solely responsible for the regulated biosynthesis and release of the hormone insulin into the bloodstream to maintain blood glucose homeostasis and whole body metabolism at normal/healthy levels. Despite extensive imaging-based investigations of the β -cell at both the light microscope (LM) and electron microscope (EM) levels over the past four decades – and, more recently, using state-of-the-art magnetic resonance (MR) and positron emission tomography (PET) techniques to image pancreatic islets both in vivo and in vitro – many fundamental questions still remain unanswered regarding how structure–function relationships change in the β -cell under different physiological conditions and how such changes contribute to the onset/progression of β -cell dysfunction and/or death. To address these key questions, we have pioneered a number of novel approaches based on the imaging method known as “electron tomography” (ET) to computationally reconstruct and analyze large cellular volumes (“tomograms”) for immortalized insulin-secreting cell lines as well as β -cells imaged in situ in fast-frozen pancreatic islets. High (~ 5 nm) resolution 3D reconstructions of the Golgi region and whole cell tomograms of islet β -cells reconstructed at intermediate (~ 10 – 20 nm) resolution have allowed us to quantitatively map the organization and morphometry of key organelles/compartments of the insulin pathway as well as the microtubule cytoskeleton in 3D at the ultrastructural level. New computational tools developed in parallel that afford significant improvements in terms of both the speed

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and the accuracy with which islet β -cells can be reconstructed and annotated in toto in 3D now provide a powerful “whole cell mapping” approach for undertaking comparative structure–function studies of islet β -cells at the nanoscale and stand poised to provide unique insights into the β -cell as a model for the study of complex systems biology in situ in 3D in an appropriate physiological and spatial context.

Keywords Imaging · Electron tomography · 3D reconstruction · Insulin granules · Segmentation · Golgi · Mitochondria

Abbreviations

3D	three dimensions/-dimensional
ARC	Australian Research Council
2D	two dimensions/-dimensional
4D	four dimensions/-dimensional
NLM	National Library of Medicine
NIH	National Institutes of Health
CT	computed (axial) tomography
MR	magnetic resonance
LM	light microscope/microscopic/microscopy
PET	positron emission tomography
TEM	transmission electron microscope/microscopy
DMSO	dimethyl sulfoxide
PEG	polyethylene glycol
PG	propylene glycol
EG	ethylene glycol
SEM	scanning electron microscope/microscopy
EM	electron microscope/microscopic/microscopy
ET	electron (microscope) tomography
ER	endoplasmic reticulum
keV	kiloelectron volts
TGN	<i>trans</i> -Golgi network
MAM	mitochondrial-associated ER membranes
CCD	charge-coupled device
SNR	signal-to-noise ratio
GUI	graphical user interface
ERGIC	ER–Golgi intermediate compartment
VTC	vesicular–tubular clusters
RP	reserve pool
RRP	readily releasable pool
GERL	Golgi, endoplasmic reticulum, lysosome
TIRF	total internal reflection fluorescence

8.1 General Introduction

The basic study of human form and function has underpinned the establishment of modern medicine and is well recognized as having evolved in large part from the original detailed topographic drawings of human anatomy by Leonardo da Vinci, who firmly believed in the power of imaging and visualization as a critical tool for providing key insights into nature and human health. A more recent iteration of that same fundamental concept is evidenced by the National Library of Medicine's "The Visible Human Project[®]", in which the combination of a number of powerful 3D clinical imaging techniques with modern computing methods resulted in complete 3D atlases of the human body at unprecedented resolution [1, 176, 178] (www.nlm.nih.gov/research/visible/visible_human.html). Collectively, these detailed "3D maps" of the entire human body now serve as a US national resource in their own right and serve as the *in silico* platform(s) of choice for medical education, surgical training and human simulations [164, 165, 177, 179, 180]. Furthermore – and not surprisingly in light of spectacular scientific/technological accomplishments such as this – "imaging" has been highlighted by the National Academy of Engineering in the USA as one of the greatest engineering achievements of the 20th century (www.greatachievements.org), in part due to the fact that the remarkable capacity of modern 3D imaging technologies to map biological structures and processes with unprecedented precision and minimal ambiguity across a range of scales (spanning several orders of magnitude) has extraordinary potential for revolutionizing the diagnosis and treatment of human disease [29].

We and others have envisioned an important biomedical goal of accomplishing the same task for mammalian cells, whose complete structure – when mapped in 3D at high resolution – will similarly inform a wide range of efforts from basic biomedical research to public health and beyond [102, 104, 130]. Like Leonardo, we [and many others from a diverse array of scientific fields; see Chapter 1, this volume; also [161]] have been inspired by both the inherent beauty and the underlying 3D complexity of cellular structure that were revealed in our inaugural high resolution tomograms of mammalian (insulin-secreting) pancreatic cells using a method commonly referred to as cellular electron tomography (ET), even though our early efforts to image and then reconstruct portions of the β -cell in 3D represented at most approximately 1% of the cell's total volume [105–107]. However, despite the potential to provide profound advances in our understanding and appreciation of how basic cell structure varies in health and disease by mapping and visualizing the organization of mammalian cells at high resolution in 3D (particularly in the context of their normal physiological setting in tissue), the generation of 3D atlases for whole mammalian cells at the organelle level has – until very recently – simply not been feasible from a technical standpoint. Driven by this challenge, over the past few years we have pioneered two separate approaches that not only demonstrate that this ambitious task is now plausible but afford us a unique opportunity to map and compare changes in organelle (esp. mitochondria) structure and function for multiple cells exposed to different conditions that both reflect normal physiology and mimic the pathophysiology of chronic diseases such as diabetes.

Since the first images of cultured mammalian cells recorded to film using an electron microscope (EM) were published in the United States in 1945 [158], the ultrastructural analysis of mammalian cells/tissues has relied almost entirely on extrapolating the three dimensionality of the structures/compartments under investigation from sets of two-dimensional (2D) images [102, 104]. Indeed, much of our current understanding of islet/ β -cell biology stems from a plethora of elegant ultrastructural studies undertaken predominantly since the late 1960s (see Section 8.2). More recently, ET – often termed “cellular tomography” when used to reconstruct 3D image volumes of cells rather than isolated organelles or macromolecules – has emerged as a powerful research tool for studying mammalian cell/tissue biology in situ in 3D in an appropriate physiological and spatial context, using cells/tissues that have been either “cryo-immobilized” in a native “frozen-hydrated” state or embedded in plastic (“resin”) following chemical- and/or cryo-fixation [47, 87, 88, 90, 115, 116, 152, 175]. ET uses mathematical techniques to computationally reconstruct a 3D density distribution (i.e. a “tomogram”) from a set of 2D digital images of an object viewed in different orientations/from different angles, in a similar manner to 3D clinical/diagnostic imaging techniques such as computed (axial) tomography (CT), magnetic resonance (MR) imaging and positron emission tomography (PET) [29, 83, 101, 109, 117]. However, rather than reconstructing part of a person’s (or an animal’s) anatomy in 3D (e.g. a CT scan), cellular ET instead results in the generation of 3D image volumes that reveal part (or even all) of a cell’s anatomy, but at approximately 10^5 – 10^6 times higher resolution (i.e. ~ 4 – 10 nm) than for the clinical tomographic imaging approaches listed above [29, 81, 85, 159].

8.2 Background to Methods and Rationale

8.2.1 Introduction – *Same Dog, New Tricks*

Most of what we currently accept as conventional wisdom regarding structure–function relationships among key organelles of the insulin secretory pathway came out of the large number of conventional (mostly 2D) EM studies of pancreatic islets/ β -cells that were undertaken mainly throughout the 1970s and 1980s [10, 11, 12, 61, 68, 69, 70–72, 138, 139, 141, 142–144]. However, numerous disparities emerged (and still continue to surface) between some of the fundamental concepts/models derived from those studies and newer insights into the molecular mechanisms underpinning and/or regulating these events that have been afforded subsequently through rapid advances in a variety of complementary methods (e.g. live cell imaging, molecular biology and computational biology), including the ever-increasing array of “-omics” approaches [4, 30, 58, 59, 96, 147, 160, 168, 171, 182, 185]. In particular – and by way of example – precise ultrastructural images detailing the mechanisms and machinery involved in the exocytosis, docking and fusion of insulin granules at the cell surface in glucose-stimulated islet β -cells remain lacking [136]; certainly, very little data are available in the published literature characterizing these processes at high spatial resolution (i.e. on the nanometre scale)

and in 3D, with the notable exception of a handful of elegant freeze-etching studies in the 1970s and 1980s [21, 137, 140] (see also Section 8.3.2 and Chapters 2, 5, 21 and 23). The absence of such fundamentally important data is perplexing, given the enormous potential of such information to provide key insights into critical aspects of insulin granule exocytosis underlying the first (rapid) versus second (sustained) phases of insulin release, especially regarding the exact nature and availability of different granule subsets or “pools” for recruitment and release [36, 135, 136, 181], as well as the involvement of the cytoskeleton [15, 28, 63–68, 74, 78, 100, 123, 125, 136, 174, 188, 194], SNARE proteins and cytosolic $[Ca^{2+}]$ stores in regulating these events [184].

Thus, in 1998 we set out to develop methods for the improved ultrastructural preservation and 3D imaging of β -cells, with a view to being able to visually characterize and precisely quantify the cellular machinery involved in the synthesis, processing and secretion of insulin with unprecedented reliability and resolution. The logic behind this was straightforward. Dramatic improvements which had occurred since the late 1980’s in terms of the development of advanced cryo-preparative methods for EM [31, 50, 112], new instrumentation for ET [82, 83, 87, 102] and dedicated software for the 3D reconstruction/analysis of complex biological data [46, 84, 109] meant that new structural tools were finally in hand to provide fresh insight into the mechanisms underlying insulin biosynthesis, trafficking and exocytosis in the β -cell.

Consequently, over the past decade or so, and almost four decades after Keith Porter’s original vision of mapping 3D structural landscapes inside mammalian cells from sets of 2D images of them taken at different tilts using dedicated high-powered microscopes, we have developed a technical pipeline that now allows us to rigorously pursue 3D structural studies of β -cell organization at the organellar, cellular and (most recently) macromolecular scales, to provide quantitative structural data on the insulin pathway in β -cells for different physiological states. By necessity, this work has led us to develop and/or advance techniques for the improved ultrastructural preservation and 3D imaging of β -cells, first for immortalized (rodent) insulin-secreting cell lines [105, 107] and then for β -cells cryo-preserved in situ within intact pancreatic islets isolated from either mice or humans [106, 132]. Our technical approach has centred on combining methods for (1) cooling β -cells/islet tissues to -196°C near instantaneously (i.e. within milliseconds) to capture β -cell/islet physiology in a vitrified, “close-to-native” state, (2) low-temperature cell/tissue processing (“freeze-substitution”), (3) high resolution 3D imaging of large cellular volumes using novel ET approaches [101, 107, 130] and (4) more efficient/accurate annotation and analysis of cellular tomograms using new algorithmic/computational tools for extracting useful and quantitative structural/biological information from large tomographic image volumes as quickly and reliably as possible, and in a semi-automated manner [106, 130–132, 192].

The reasoning behind development/application of the various methodological advances that collectively underpin our current pipeline for mapping β -cells at the nanoscale in 3D is discussed throughout the remainder of this chapter, supported by some examples selected to highlight recent improvements in our methods for whole

cell tomography and to illustrate some of the novel insights into β -cell/islet biology that have come via this approach which could not have been otherwise attained.

8.2.2 Initial Method Development for Improved Ultrastructural Preservation – Making β -Cells Even Cooler

With only a handful of exceptions [32–35, 62], ultrastructural studies aimed at mapping the spatial/structural organization of pancreatic islets/ β -cells over the past four decades have employed conventional chemical fixation methods to stabilize pancreatic/islet tissue or isolated/primary β -cells for visualization in the EM. However, as this process takes anywhere from seconds to several minutes, dynamic events and/or subcellular structures that are highly labile are unlikely to be reliably preserved. Certainly, serious concerns have been raised in the past “that [conventional chemical] fixation can only fix the response of the cell to the fixative (and not the original state) [of the cell]” [32, 50, 62, 155].

Thus, with the aim of capturing β -cell structure and function in a more optimal “near-native” state prior to imaging and 3D reconstruction by high resolution ET, we initiated our structural studies of the β -cell using a number of well established, immortalized β -cell lines derived from rodents (e.g. HIT-T15, MIN-6, INS-1 and β TC-3) [37, 120, 156, 173]. In addition to (mostly) retaining their capacity to synthesize and secrete insulin in response to elevated levels of extracellular glucose (i.e. at least at early passage number) [157], the fact that immortalized β -cells cultured in vitro typically contain far fewer granules than do their islet counterparts either in vivo or in vitro provides an advantage of sorts for EM-based studies of the insulin secretory pathway, since interactions between the cytoskeleton and relevant subcellular structures can be more clearly visualized [42, 65, 105, 134, 200, 201] (Fig. 8.1; see also Fig. 8.7).

Of the various “fast-freezing” methods that were tested, the technique referred to as “high pressure freezing” reproducibly yielded the best results in terms of the quality of ultrastructural preservation for all four of the β -cell lines examined, which further suggested the utility of this method for plans to subsequently extend the approach to freshly isolated mouse and human islets. High pressure freezing is just one of a number of methods for rapid cryo-fixation that can be used to “vitrify” cells/tissues, thereby immobilizing all cellular processes within milliseconds; in this instance, the term “vitrification” refers to the process of cooling a fluid so quickly that it transitions directly from a liquid to a solid “glass-like” state (i.e. a special form of ice) without the formation of crystalline ice; extensive “freeze-damage” to cells results from the nucleation and rapid expansion of ice crystals intracellularly. Typically, true vitrification of “unprotected” cells/tissues requires cooling at rates estimated to be on the order of $>10,000^{\circ}\text{C/s}$ [26, 50, 122], which in practice can be attained only with specimens of limited thickness (e.g. 10–20 μm). However, high pressure itself acts as a cryo-protectant for cells/tissues by lowering the melting point of water; this reduces the rate of ice crystal nucleation, thus allowing vitrification to be achieved despite much slower cooling rates (on the order

of 100–500°C/s) [26]. Thus, high pressure freezing is better suited to fast-freezing comparatively thick specimens (up to $\sim 600\ \mu\text{m}$) such as isolated islets without detectable ice crystal damage at the ultrastructural level [40, 50]. When employed in combination with “freeze-substitution”, high pressure freezing can provide dramatic improvements in the preservation of cell/tissue ultrastructure (particularly in cells, tissues and organisms that are typically difficult to fix reliably for study by EM using conventional methods) yet still permits subsequent embedding in plastic/resin for processing at ambient temperatures and post-staining to improve image contrast in the EM [112, 113, 116, 117].

Given the focus of this text on studies of β -cell biology, it is thus important to distinguish here between this use of the term “vitrification” and the same term used routinely in the field of tissue cryonics/cryo-biology, especially with respect to the numerous studies in which cryo-preservation/vitrification has been investigated for improving the viability of isolated human islets thawed following long-term storage at cryogenic temperatures [8, 75, 76, 77, 92, 146]. In the case of cryonics, however, although vitrification also refers to the process of solidifying a fluid by increasing the viscosity of the liquid by “rapid” cooling, the cooling times referred to as “rapid” are at least 1–2 orders of magnitude slower ($<10^\circ\text{C}/\text{minute}$) than for the methods described above. Thus, these procedures typically require the addition of very high concentrations of intracellular cryo-protective agents [e.g. dimethyl sulfoxide (DMSO), polyethylene glycol (PEG), propylene glycol (PG), ethylene glycol (EG), acetamide, glycerol, raffinose, sucrose] [8, 41, 53, 77, 92]. Although these cryo-protectants help to minimize the extent of freeze-damage that occurs, many are themselves quite toxic as well as osmotically active and thus cause any number of detrimental effects on cells such as osmotic disruption and swelling, hypothermic stress responses during slow cooling and apoptosis [50, 91, 154].

8.2.3 Tomographic Reconstruction of β -Cells – 3D Snapshots of the Insulin Pathway Literally “Frozen in Time”

As outlined above, we have focused our high resolution 3D characterization of the β -cell on cells/tissues preserved by fast-freezing to ensure that the resulting 3D reconstructions reflect the physiological state and architecture of the β -cell as accurately as possible prior to preparation and/or imaging in the EM. Over recent years, high resolution ET studies of frozen-hydrated cells (i.e. mostly prokaryotes and simpler eukaryotes) directed by Wolfgang Baumeister at the Max Planck Institute of Biochemistry (Martinsried, Germany) in particular have generated considerable enthusiasm around the globe for the ambitious concept of mapping macromolecules in situ in 3D in their correct cellular context and in a near-native state [5–7, 9, 43, 54, 86, 118, 127]. Indeed, cellular tomograms of this kind have been described as “3D images of the entire proteome of the cell” [43, 172], and the term “visual proteomics” has been aptly coined [128]. Furthermore, such efforts

have already drawn significant attention from the systems biology and bioinformatics communities because of the obvious/inherent value that such precise spatial data have for uniquely informing *in silico* studies of cell structure and function [14, 94, 128].

Unfortunately, however, most mammalian cells are simply too large to be imaged intact at high resolution in this manner. Instead, mammalian cells/tissues prepared by fast-freezing must typically be sliced into “sections” thin enough to be viewed in the EM. However, since cutting sections directly from frozen-hydrated cells and subsequently imaging them under cryogenic conditions at low dose by transmission EM (TEM) encompasses a number of considerable technical challenges [2, 3, 57, 73, 89, 95, 114, 126, 153], a more practical approach – and the one that we have employed for our ET studies of the β -cell – involves first processing frozen-hydrated β -cells/islet tissues for EM at ultra-low temperatures by “freeze-substitution” as noted in the preceding section, followed by infiltration with plastic/resin. Once embedded in plastic and polymerized, the cells/tissues can then be sliced (relatively) easily into semi-thick sections (~ 250 – 400 nm) for subsequent imaging by EM (Fig. 8.1).

Although in most instances sections are viewed/imaged individually for ET, serial section tomography represents an extension of the basic approach that enables the 3D reconstruction and analysis of much larger cellular volumes without sacrificing resolution, by aligning and then joining serial section tomograms sequentially in *Z* to eventually compute a single large volume [88, 101, 106, 107, 175]. Large cellular volumes reconstructed in this manner have provided a practical workaround to overcome the physical limitations of specimen thickness for imaging in the TEM and have recently enabled the detailed 3D characterization of extended subcellular



Fig. 8.1 Overview panel illustrating relative differences in image quality between tomograms of large subvolumes within insulin-secreting cells reconstructed at high resolution versus tomograms of entire β -cells reconstructed at intermediate resolution by cellular ET. **(a)** A pixel-thick image slice (*XY* view) is shown from a high resolution (~ 6 nm) dual-axis tomogram of the Golgi region in a HIT-T15 cell. Scale bar: 500 nm. **(b)** Stacked Golgi cisternae in the centre of the region are displayed in the context of neighbouring structures. Colour coding: Golgi cisternae (*cis-trans*, light blue, pink, cherry, green, dark blue, gold, red); ER (yellow); membrane-bound ribosomes (blue); free ribosomes (orange); microtubules (bright green); insulin granules (blue); clathrin-negative vesicles (white); clathrin-positive compartments/vesicles (red); clathrin-negative compartments/vesicles (purple); mitochondria (green). **(a** and **b)** Reproduced from data originally published in [107]. **(c** and **e)** Image slices (*XY* view) from whole cell tomograms of two islet β -cells reconstructed at intermediate resolution (~ 15 – 20 nm). Colour coding: Golgi (main ribbon/*cis*-medial cisternae, grey; *trans*-most cisternae, red); mitochondria (green); mature insulin granules (dark blue); immature insulin granules (light blue); nucleus (yellow); plasma membrane (purple/pink). Higher magnification views of $1\ \mu\text{m}^2$ areas from each tomographic slice displayed in the insets reveal the relative clarity of Golgi membranes for comparison with **(a)**. **(d** and **f)** The size, number and distribution of the insulin granules in each β -cell were determined by quantitative analysis of the 3D models derived by segmenting the whole cell tomograms. Colour coding: mature insulin granules (dark blue); immature insulin granules (light blue). **(c–f)** Reproduced from data originally published in [130]. Scale bar: 1000 nm.

structures such as the endoplasmic reticulum (ER), the Golgi ribbon and the microtubule cytoskeleton at the cellular scale [60, 98, 130]. In contrast to the severely limited resolutions obtained in past tomographic reconstructions of whole cells imaged intact in the EM [124, 151], image slices from 3D cellular reconstructions

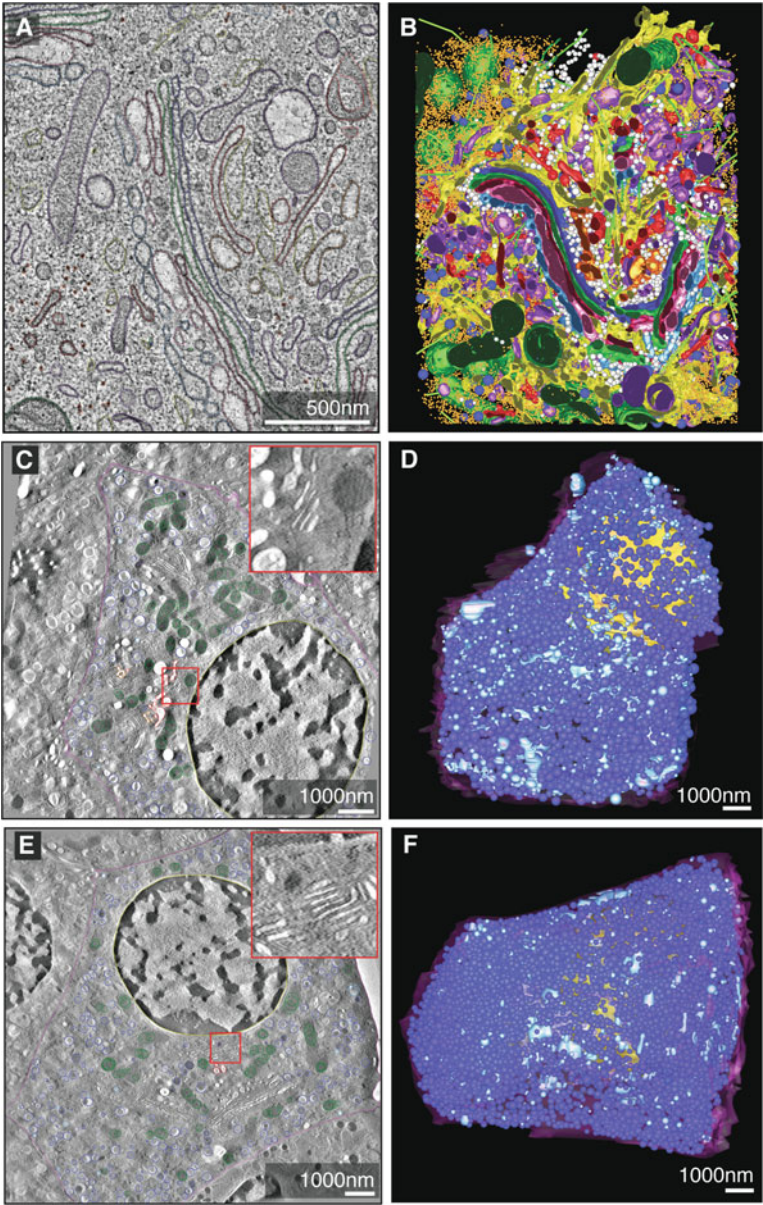


Fig. 8.1 (continued)

generated using a serial section ET approach demonstrate sufficient quality to permit finer structural information relevant to studies of insulin trafficking/granule exocytosis, such as protein coats (e.g. clathrin) as well as cytoskeletal structures such as microtubules and intermediate filaments, to be distinguished with minimal ambiguity [88, 101, 102, 105, 107, 175]. Nevertheless, it should be noted that whole cell tomography using a serial section approach presents considerable technical challenges. Our own recent efforts to generate tomograms of entire islet β -cells at intermediate resolution (~ 15 – 20 nm) required collecting large numbers of 2D images for many dozens of serial (semi-thick) sections, from which individual tomograms reconstructed for each section were then aligned/assembled *in silico* to allow computation of a final volume that encompassed the cell *in toto* [130] (Figs. 8.2 and 8.3). Application of this whole cell tomography approach for mapping the 3D organization of islet β -cells *in situ* with high spatial precision, as well as some of the recent modifications that have afforded significant improvements in both speed and accuracy, will be discussed in more detail below following a brief introduction of the basic principles of cellular tomography as we have applied them for our 3D studies of the β -cell.

In general, the basic approach for cellular ET requires collecting sets of 2D images using an EM that operates at higher-than-normal voltages (e.g. ≥ 300 keV compared to conventional EMs that operate in the range of 80–120 keV), as the section is serially tilted by small, regular increments (e.g. 1° or 1.5°) over a relatively large angular range (e.g. ± 60 – 70°). Such sets of images – referred to as a “tilt-series” – are first aligned with one another by mathematically “cross-correlating” information that is common from one image in the series to the next in order to bring them into registration with one another. Subsequently, the images are more accurately aligned by tracking the positions of small (10–15 nm) gold fiducial markers adhered to both surfaces of each section prior to imaging in the EM, and a tomogram is computed for each tilt-series collected around a single axis by “weighted back-projection”. For high resolution cellular tomography, tilt-series data are frequently collected around two orthogonal axes rather than just a single axis. Because the resolution of an object within the plane of a specimen with a slab geometry (i.e. a semi-thick section cut from plastic-embedded cells/tissues) also depends on its orientation relative to the axis around which the specimen is tilted, the so-called dual-axis approach produces tomographic reconstructions that demonstrate

Fig. 8.2 Views along the XZ-axis from whole cell tomograms of islet β -cells reconstructed at intermediate resolution(s) using either a conventional “single-axis” approach or a “reduced dual-axis” approach. (a) Despite the inherent limitations imposed on tomogram quality/isotropy outlined in this chapter, the whole cell tomograms reconstructed/assembled for the two islet β -cells characterized in [130] deliberately employed single-axis data only to expedite image acquisition and tomogram reconstruction/analysis (Fig. 8.1c, e). (b) Substantial modifications to our data collection/processing strategy for whole cell tomography (illustrated in Figs. 8.3, 8.4, and 8.5) have afforded significant improvements in tomogram accuracy and image quality for equivalent 3D reconstructions of islet β -cells generated subsequently. Scale bar: 1000 nm.

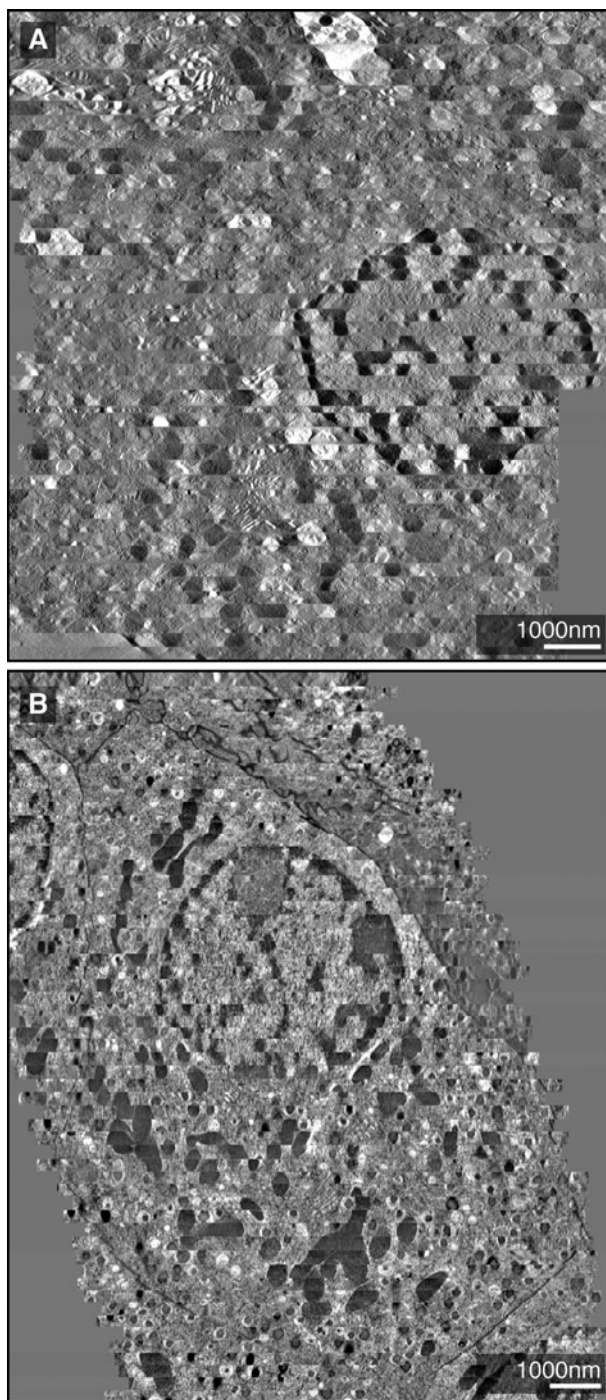


Fig. 8.2 (continued)

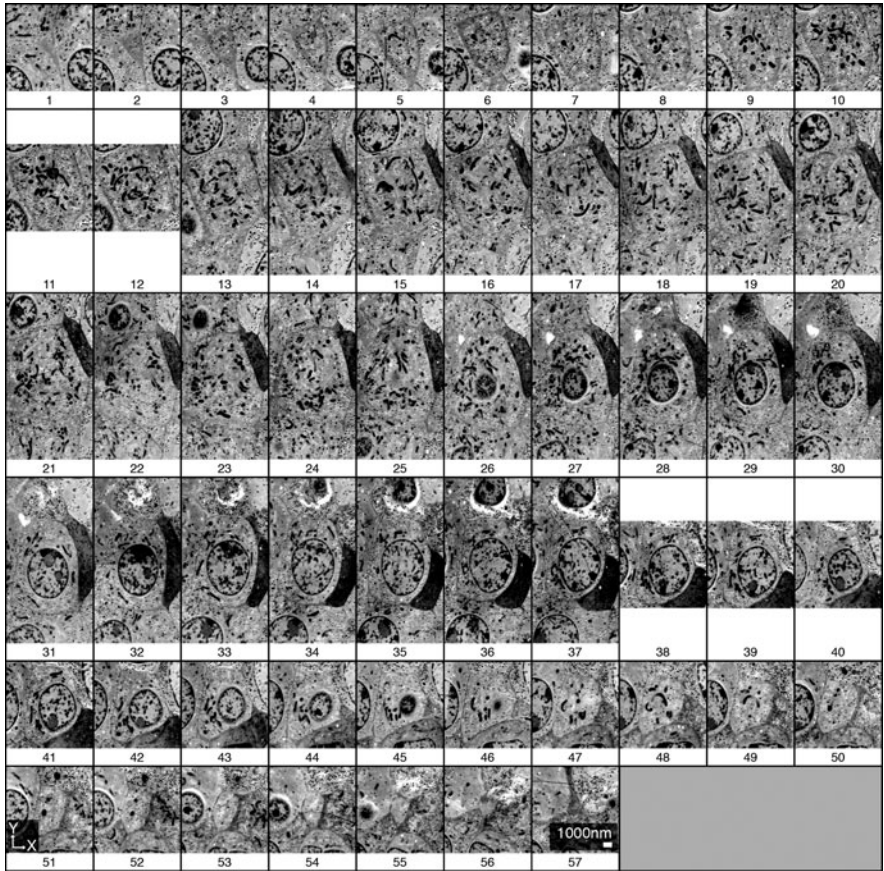


Fig. 8.3 3D reconstruction of islet β -cells in toto by sequential acquisition, alignment and assembly of “tilt-series” using an improved whole cell tomography approach that incorporates 2D image montaging. 2D images taken at 0° tilt are shown from each of the tilt-series datasets acquired from a set of serial semi-thick sections (~ 300 nm) encompassing an islet β -cell in its entirety. Tilt-series were collected around two orthogonal axes for each section using a modified dual-axis approach (see Fig. 8.4); note that 0° views from only one of each pair of tilt-series are presented. In the example presented here, tilt-series images for sections 13–37 were acquired as montaged panels (1×2 fields of view on the CCD camera) to fully accommodate the different dimensions of the β -cell in cross-section (i.e. in XY) at different depths through the islet (i.e. along the Z -axis). A visible seam evident in some of the panels highlights the zone of overlap ($\sim 10\%$ of the pixel dimensions of the CCD field of view) used to precisely stitch image montages together in XY by cross-correlation to produce a single “blended” image prior to tilt-series processing for tomogram computation. Scale bar: 1000 nm.

improved symmetry and resolution in all three dimensions [109]. In this case, individual tomograms generated from tilt-series data collected around each axis are brought into register and then mathematically combined in 3D space to produce a single volume (i.e. a dual-axis tomogram) that integrates spatial information from each of the component single-axis tomograms (Fig. 8.1a, b).

8.2.4 Seeing Is Believing – But Only If You Can Make Sense of It All

The sheer scale as well as visual and structural complexity of the image volumes produced by cellular tomography means that significant effort is required in terms of image analysis/annotation before quantitative analyses can be undertaken and new biological insights obtained. However, the relatively low signal-to-noise ratio (SNR) of volumetric image data generated by ET of islets/ β -cells prepared using the methods outlined above has meant that the process of accurately defining basic features within the image slices (“segmentation”) is difficult, somewhat subjective and time-consuming [102, 148, 197]. Unfortunately, attempts at automatically segmenting cellular tomograms using algorithmic approaches have to date achieved only limited success [44, 45, 49, 106, 149, 150, 166, 192, 197]. Instead, large cellular volumes reconstructed by ET, such as the examples presented in Fig. 8.1a, c, e, have for the most part required manual segmentation by a trained biologist(s), using tools available in dedicated software packages such as *IMOD* [84] to draw line segments (“contours”) defining each compartment/filament on every [pixel-thick] tomographic slice that the structure spans in *Z*. Nevertheless, once all structures of interest have been segmented, 3D surface meshes generated from the stack of 2D image contours for each spatially distinct organelle/compartment (and/or other structures such as ribosomes and microtubules) are then available for 3D visualization either by themselves or together with any combination of object(s), as well as for quantitative analysis and functional annotation (Fig. 8.1b, d, f).

8.2.5 Reconstructing the Insulin Pathway in 3D – ET Studies Using Transformed β -Cell Lines Versus Islet β -Cells

Our early efforts to structurally characterize how insulin is processed and packaged at the Golgi by cellular tomography using insulin-secreting cell lines seemed like a logical jump-off point (see also Section 8.2.2), since immortalized β -cell lines continue to provide useful cell culture models for basic investigations of the insulin pathway and β -cell biology [17, 51, 96, 157]. Despite the fact that numerous EM studies to investigate the 3D structure of the Golgi and related membrane traffic compartments involved in both constitutive and regulated secretion had been undertaken for other cell types [24, 79, 87, 162, 163, 186, 187], no major 3D structural study of the Golgi had been undertaken at the EM level for the β -cell.

The high resolution tomogram of the Golgi region which emerged from our inaugural 3D studies of the β -cell (Fig. 8.1a, b) – generated by serial section tomography of three serial semi-thick (~ 400 nm) plastic sections cut from an insulin-secreting HIT-T15 cell that had been imaged following steady-state culture in vitro in the presence of 7 mM glucose – was at the time the largest volume ($3.1 \times 3.2 \times 1.2 \mu\text{m}^3$) ever reconstructed for any mammalian cell at such high resolution [105, 107]. However, although those data provided a number of fundamental new insights

into structure–function relationships at the Golgi underpinning insulin transport in particular, and protein trafficking in mammalian cells more generally [102, 103, 105, 107, 121], there are/were obvious limits to which biological conclusions drawn from studies of transformed β -cell lines can be reliably extrapolated to the in vivo situation. Insulin-secreting cell lines tend to contain far fewer insulin granules (and thus contain significantly lower amounts of insulin), are less responsive (in terms of granule exocytosis) to stimulation with glucose at physiologically relevant concentrations and generally fail to exhibit bona fide biphasic patterns of insulin granule release compared to islet β -cells either in vivo or in vitro [17, 156, 195]. Taken together with the fact that islet β -cells are polarized in situ, whereas transformed β -cell lines generally are not [4, 12, 17, 25, 145, 199], it remains somewhat unclear if the mechanisms that immortalized β -cells have retained for sorting, processing and trafficking insulin in vitro still accurately reflect the equivalent steps in islet β -cells, since the loss of cell polarity is known to have substantial effects on protein trafficking pathways [97].

Consequently, not long after that initial study was published, we moved quickly to adapt the combination of fast-freezing/freeze-substitution with high resolution dual-axis ET of large cellular volumes for work with pancreatic islets isolated from adult female Balb/c mice (as well as NOD, C57BL/6 and various transgenics on these backgrounds), and were successful [101, 102, 106, 108, 130].

8.2.6 Whole Cell Tomography for Mapping Islet β -Cell Biology In Toto in 3D at The EM Level – Getting The “Bigger Picture”

Despite the importance of being able to generate high resolution 3D reconstructions of large volumes within islet β -cells for a range of physiological conditions and time points following different experimental treatments (e.g. secretagogue stimulation), each such tomogram still represents only a narrow cross-sectional slice through a cell that we estimate represents $\leq 1\%$ of a typical islet β -cell's volume. Thus, several years ago we moved towards a more integrated and holistic approach to understanding the β -cell as a primary example of complex system by pioneering new ET methods for imaging and reconstructing islet β -cells in their entirety in 3D to provide important information on key compartments of the insulin biosynthetic/secretory pathway, accompanied by the development of more efficient yet accurate approaches for segmenting or “marking up” 3D data sets at the cellular scale.

The “whole cell tomography” approach that we developed resulted in fully annotated 3D reconstructions of entire islet β -cells within just months [130], albeit at lower resolutions (~ 10 – 20 nm) than we normally use for 3D studies of insulin trafficking in the Golgi region in β -cells [39, 106–108]. As noted briefly in Section 8.2.3, whole cell tomograms were generated by sequentially aligning and then joining (i.e. in Z) individual tomographic volumes imaged from multiple serial sections. In comparison to the three serial sections required to generate the high resolution

tomogram of the Golgi region in the HIT-T15 cell shown in Fig. 8.1a, b, assembly of tomographic volumes sufficiently large to encompass the two β -cells shown in Fig. 8.1c, e in their entirety in 3D required serial section reconstruction and alignment of 45 and 27 tomograms, respectively, as demonstrated in Fig. 8.2 and also illustrated in Fig. 8.3. Like our previous tomography studies using β -cell lines, individual tomograms had to be computed from tilt-series collected from semi-thick (~ 300 nm) sections cut from a plastic-embedded mouse islet imaged after stimulated *in vitro* with elevated (11 mM) glucose for ~ 1 hour, following pre-culture for 12–18 hour under steady-state/normoglycemic conditions (i.e. 5.6 mM glucose) post-isolation [130]. For reference, the whole cell reconstructions shown in Fig. 8.1c, e encompassed 1529 and 1362 μm^3 (i.e. $10.6 \times 10.6 \times 13.7$ and $12.7 \times 12.7 \times 8.4$ μm^3) and were represented by a total of 2217 and 1153 digital image slices, respectively, compared to our inaugural high resolution (~ 6 nm) 3D reconstruction of the Golgi region in the HIT-T15 cell (Fig. 8.1a, b) which encompassed 11.9 μm^3 (represented by 315 digital image slices) [105, 107]. On this point, it is worth reiterating that at the time the latter effort constituted perhaps the single largest 3D reconstruction of a mammalian cell at high resolution by ET and was described as an “heroic” achievement with the potential to have “high impact on how we imagine life in the cell” [5, 48, 110, 116].

Although our initial 3D reconstructions of whole islet β -cells have likewise mustered considerable enthusiasm for mapping the 3D landscape in the β -cell through ongoing use of this approach, the quality of those data suffered due to a number of constraints that we imposed deliberately to ensure a practical compromise between tomogram quality/accuracy and the efficiency of image data acquisition and tomogram reconstruction/analysis at the whole cell scale. These limitations included a requirement to (1) collect tilt-series datasets at magnifications (i.e. 4700 $\times$ and 3700 $\times$, respectively) much lower than those normally used for the kinds of high resolution ET studies highlighted earlier in this chapter, and (2) acquire tilt-series images for tomogram computation for only a single axis, which, although has represented “convention” in the field for many groups with expertise in cellular ET, was in stark contrast to the standard dual-axis approach employed for our high resolution 3D studies (Fig. 8.2a). Nevertheless, it was readily apparent that despite these handicaps, this expedited ET method still yielded precise, quantitative and unique information regarding the 3D organization of islet β -cells *in situ* that simply could not be obtained by other imaging methods and at 1–2 orders of magnitude higher resolution than the typical kinds of LM imaging approaches used by most laboratories. For example, the data obtained in these initial whole cell studies afforded direct visualization and precise quantitation of the number, size and distribution of the entire pool of insulin granules in each cell (as illustrated in Fig. 8.1d, f) and revealed that one of the two cells (Fig. 8.1d) contained significantly fewer mature insulin granules (3370; average diameter 280 nm) compared to its sibling β -cell from the very same glucose-stimulated islet (Fig. 8.1f), which contained 8250 mature granules (average diameter 245 nm).

More recently, we have developed an improved strategy for tomography of entire islet β -cells that has significantly bolstered both the efficiency and the accuracy

of whole cell image reconstruction in 3D (Fig. 8.2b) by incorporating a number of substantial modifications. Among these improvements is the use of 2D image montaging during tilt-series acquisition (illustrated in Fig. 8.3), which we have employed extensively in the past and present for high resolution ET of islet β -cells [101, 106]. Based on our original whole cell reconstructions of islet β -cells [130], we had determined that tilt-series data for whole cell tomography should ideally be acquired at a minimal magnification of $4700\times$ to provide sufficient resolution for reliably distinguishing key subcellular structures involved in insulin trafficking. Since the orientation of islet β -cells as viewed in plastic sections in the EM is random, and due to natural variations in size observed for individual β -cells, the capacity to use digital montaging when collecting tilt-series data sets ensures that the dimensions of any given β -cell can be accommodated in cross-section during imaging, without having to use a lower magnification (Fig. 8.3). A second major improvement originally proposed in [130] stems from incorporating a modification of the standard dual-axis approach (highlighted in Fig. 8.4), which

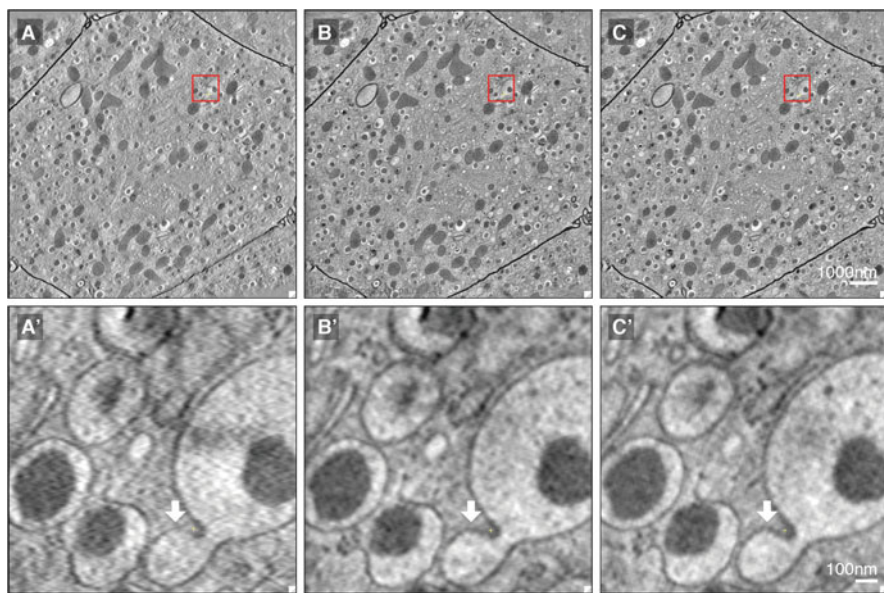


Fig. 8.4 Modification of the conventional dual-axis approach for whole cell tomography of islet β -cells. Incorporating a limited set of tilts from a second axis affords improved tomogram accuracy and image quality. Image slices taken from the mid-plane (in Z) of tomograms generated from the same primary tilt-series data taken at $4700\times$ magnification (1.5° increments, $\pm 63^\circ$) are shown at both low (a–c) and high (a'–c') zoom, respectively, for comparison. (a and a') The middle slice from the tomogram generated using a conventional “single-axis” approach. (b and b') The middle slice from the tomogram generated using a “reduced dual-axis” approach. (c and c') The middle slice from the tomogram generated using a conventional “dual-axis” approach. Any structures that were originally oriented perpendicular to the tilt axis are poorly resolved in the single-axis tomogram (a and a') compared to either of the tomograms utilizing data from a second axis, as highlighted by the arrows. Scale bars: (a) 1000 nm; (c') 100 nm.

requires collecting only half as many images for the second tilt-series to compute a “reduced second-axis” tomogram. Although significant differences in image quality/information are evident when corresponding tomographic slices are compared between a standard single-axis reconstruction (Fig. 8.4a, a') versus a standard dual-axis reconstruction (Fig. 8.4c, c') generated from the same primary test dataset, differences in image quality/information content between the “reduced dual-axis” reconstruction (Fig. 8.4b, b') versus the standard dual-axis tomogram (Fig. 8.4c, c') were deemed to be negligible for data collected at this magnification (i.e. 4700 $\times$). This indicated that despite only using a limited set of tilt images to compute the

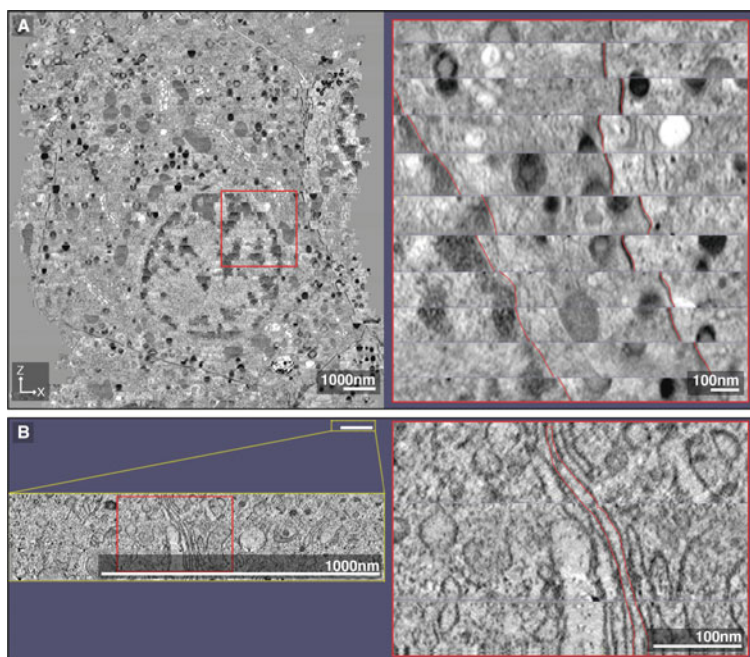


Fig. 8.5 Figure showing the relative extent of misalignment between adjacent section volumes for high resolution versus intermediate resolution 3D reconstructions of β -cells. (a) A side view through a 3D reconstruction of an islet β -cell generated using the new/improved tomographic approaches described in Figs. 8.2b, 8.3 and 8.4 reveals the misalignment that persists between adjacent section volumes after alignment despite using the same software tools/methods employed for alignment of serial section tomograms generated at high resolution, as illustrated in (b). To better illustrate the extent of misalignment between tomograms reconstructed using intermediate-versus high resolution methods, the areas boxed in red on the left have been expanded in the insets on the right. Within each inset, section boundaries are highlighted in blue, while the nuclear envelope and plasma membrane are highlighted in red to reveal discontinuity across section boundaries. (b) A side view through part of the high resolution tomogram of the Golgi region presented in Fig. 8.1a. Dotted yellow lines reflect the scale of this volume relative to the whole cell tomogram in (a). Within the corresponding inset, the membranes of a single Golgi cisterna are highlighted in red to reveal discontinuity across section boundaries. Scale bars: (main images) 1000 nm; (insets) 100 nm.

tomogram for the second axis – which is then combined with the conventional tomogram from the first axis to generate a final “modified dual-axis” tomogram as shown in Fig. 8.4b, b’ – the information contributed from those views was sufficient to result in more isotropic reconstruction and significantly reduced noise when compared to the single-axis tomogram alone.

Although not discussed here in detail, the development/application of additional technical strategies to correct for the physical changes that occur to plastic sections during imaging in the EM (e.g. deformation in Z due to the phenomenon of “section collapse” and anisotropic distortion in XY caused by “section thinning”) [16, 109] has contributed to dramatic overall improvements in image quality and spatial accuracy for more recent whole cell tomograms generated for islet β -cells under different physiological conditions (see Figs. 8.2b, 8.5a, and 8.7d) and now affords final resolutions more on the order of ~ 10 – 15 nm. Nevertheless, the fundamental limitations imposed by this approach mean that the final resolution and accuracy of the data will always be limited when compared to bona fide high resolution ET approaches (as illustrated by the example of serial section volume misalignment in Fig. 8.5). Thus, we have been actively pursuing the development of a second complementary approach for whole cell tomography in parallel that affords a genuine capacity to reconstruct islet β -cells in toto in 3D at resolutions sufficiently high to permit the in situ identification of macromolecules by virtue of their 3D structure alone. However, this latter exciting approach will for now have to remain a topic for future discussions.

8.3 “Holistic” Insights from 3D Image Reconstruction of the β -Cell at Nanometre Resolution

8.3.1 *Developing a More Integrated Understanding of Cellular Organization and Membrane Transport in Insulin-Secreting Cells*

Over half a century after the images from the seminal publication by Albert Claude, Ernest Fullam and Keith Porter had demonstrated the power of the EM to more accurately resolve cellular fine structure in mammalian cells and consequently revolutionized the study of modern cell biology forever [158], our inaugural high resolution tomogram revealing the amazing degree of spatial/structural complexity in the Golgi region imaged within part of a single HIT-T15 cell has impacted substantially on how scientists, students and the public alike picture life at the sub-cellular level in mammalian cells [5, 48, 52, 94, 116, 161]. Moreover, although the tomogram reconstructed in that study was estimated to represent just $\sim 1\%$ of the cell’s total volume (Fig. 8.1a, b), that sole high resolution 3D view of cytoplasmic organization in the vicinity of the Golgi afforded key insights into unexpected structural associations between compartments involved in insulin trafficking and provided direct evidence that the multiple transport mechanisms (i.e. cisternal progression–maturation, trafficking via membrane tubules, vesicle-mediated transport) co-existed harmoniously in the same region of the β -cell’s Golgi ribbon [103,

[105]. In addition, the study provided new data in support of older fundamental concepts that had fallen from grace [102, 103] regarding the relative roles and importance of different mechanisms for transporting cargo to, through and from the Golgi complex in the β -cell.

Among these was the idea that different types of protein cargo exit the Golgi via multiple *trans*-cisternae that are both structurally and functionally distinct – otherwise typically/collectively referred to as the *trans*-Golgi network (TGN) – along the lines of Alex Novikoff’s original “GERL” hypothesis [133]. Tomographic data obtained in support of this notion were also in close accordance with James Rothman’s original “distillation tower” hypothesis, in which it was suggested that cargo sorting within the Golgi might occur as a multi-step refinement process akin to fractional distillation [170]. This hypothesis proposed that the stacked *cis*-medial cisternae essentially serve as “trays” or “plates” within the distillation column, while the last layers – the *trans*-Golgi cisternae – receive their respective cargoes as “refined fractions” for re-distribution in the cell via different trafficking pathways (e.g. constitutive trafficking to apical and basolateral membranes, endo-lysosomal pathway or regulated secretory pathway) [169]. Indeed, the nature of both the mechanisms and cargoes specifically associated with each of the three cisternae classified as “*trans*” and revealed at high resolution in that tomogram provided direct evidence for differential cargo sorting/release to the basolateral, apical and endo-lysosomal/regulated secretory pathways, respectively, from the last three cisternae in those Golgi stacks.

Further, those data suggested an important role for the ER in regulating key events at the *trans*-Golgi, the presumptive site for (pro)insulin sorting and packaging into newly forming secretory granules [103–107, 193, 196]. It had been proposed that the close apposition of ER membranes with *trans*-cisternae might provide a direct mechanism for lipid exchange/modification at the ER/*trans*-Golgi interface that would facilitate the rapid transfer of lipids between these compartments [88, 105]. This hypothesis was based on previous studies demonstrating that mitochondrial-associated ER membranes (MAMs) exhibit higher specific activities for the synthesis of cholesterol, phosphatidylcholine, triacylglycerols and cholesterol esters than do conventional microsomal membranes (Vance, 1990), supported by our finding that specialized ER membranes in close association with *trans*-cisternae in that region of the Golgi were immediately continuous with adjacent sites where ER and mitochondrial membranes directly interacted in a striking physical manner [105, 107]. Thus, these data indirectly supported the idea that sphingolipid–cholesterol subdomains (frequently referred to as “lipid rafts”) play an important role in cargo sorting at the *trans*-Golgi (Keller and Simons, 1997; Mellman et al., 1993). Although the role of cholesterol in these events is not yet fully understood in the context of sorting to the regulated secretory pathway, more recent evidence suggests that altered cholesterol homeostasis in islet β -cells leads to intracellular cholesterol accumulation and impaired insulin secretion, which ultimately contribute to β -cell dysfunction [18, 19]; presumably such changes reflect the progressive loss of β -cell function and secretory capacity that accompanies type 2 diabetes. Moreover, the nature and frequency of direct physical interactions that were observed between the ER and membranes of *trans*-Golgi cisternae, mitochondria and endo-lysosomal compartments led us to propose that those particular sites

of direct contact/membrane apposition may represent part of an uncharacterized general mechanism for the transfer/exchange of lipids and/or inorganic ions between these organelles [102–106].

In addition, these tomograms have revealed novel insights into the nature of the spatial/structural associations between the microtubule cytoskeleton and Golgi cisternae at both its *cis*- and *trans*-poles (i.e. a hallmark of the “polarized” organization of Golgi stacks that assemble laterally to give rise to the mammalian Golgi’s ribbon-like architecture) to facilitate the transport of (pro)insulin to/from the Golgi for processing and packaging into granules for release [167]. Subsequently, our high resolution ET investigations of islet β -cells imaged in situ have provided convincing evidence that direct connections form between heterologous cisternae to serve as a “rapid transit” pathway for accommodating a wave of (pro)insulin traffic through the Golgi for packaging/release [39, 106]. In addition, our studies have identified a major role for increased lysosomal biogenesis and regulated autophagy in intracellular insulin degradation to maintain insulin content at optimal levels in islet β -cells in the face of insulin secretory dysfunction, and have called into question the reliability of total islet insulin content assays as an accurate method for assessing β -cell secretory capacity [108, 190]. Collectively, our studies have thus revealed the complexity of relationships among the Golgi, ER, mitochondria and compartments of the endo-lysosomal system [106, 107, 130] pertaining to regulated exocytosis and membrane trafficking in the β -cell that could not have been gleaned using other cell biological approaches.

8.3.2 New Lessons Learned from 3D Studies of Whole Cells – Mapping Mitochondrial Structure to β -Cell Function

Our recent mainstream studies to compare variations in membrane traffic at the Golgi complex associated with the formation of nascent secretory granules in islet β -cells that had been stimulated to make and release insulin (following exposure to elevated extracellular glucose) revealed a wide range of “apparent” structural and functional disparity in the Golgi region among numerous high-resolution tomograms from individual β -cells. This forced us to develop an innovative new approach in tomographic imaging that would allow us to both qualitatively and quantitatively assess structure–function relationships among the key organelles involved in insulin production, in the context of a whole cell [130].

An unexpected outcome, however, was that the quantitative data derived from detailed 3D analysis of mitochondrial number, diameter, length and the extent of branching – when assessed together with quantitative data obtained for the insulin granule pool and the Golgi complex – strongly suggested an inverse relationship among these organelles for the two cells analysed in that study. This finding reflected the relative differences in the levels of biosynthetic activity stimulated within each cell by glucose. Notably, the significant difference in the relative proportion of branched mitochondria per cell, and the average number of branches per branched mitochondrion between the two cells, indicated that the mitochondrial population

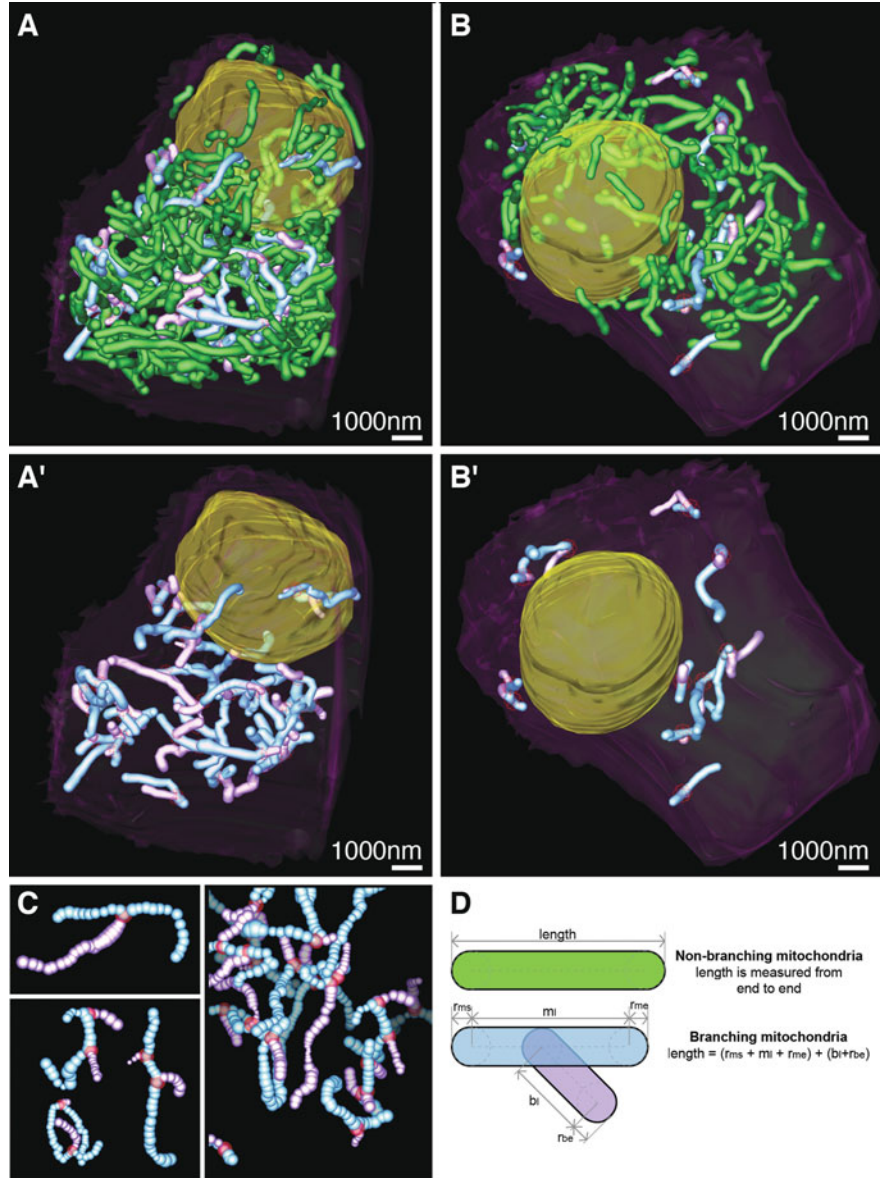


Fig. 8.6 Significant differences in the number, distribution and branching of mitochondria determined by quantitative analysis of whole cell tomograms reflect β -cell heterogeneity in response to glucose stimulation. (a and b) 3D models showing the number, size and distribution of branched versus unbranched mitochondria. Colour coding: unbranched mitochondria (green); branched mitochondria (main lengths, light blue; branches, pink; branch points, red); plasma membrane (purple); nucleus (yellow). (a' and b') The relative difference in the number of branched mitochondria in each cell is more evident when branched mitochondria are displayed alone as a subset. (c) Examples of variations in mitochondrial morphology from the 3D models displayed in (a' and b') are presented. (d) A cartoon demonstrating how mitochondrial length and branching were quantified. Scale bar: 1000 nm.

in one cell was more functionally active (i.e. exhibited a significantly higher rate of fission/fusion) than in the other [22, 23] (Fig. 8.6). A comparison of other morphometric parameters such as the average cumulative length of branched mitochondria versus non-branched mitochondria, for example, was also consistent with the idea that shorter (single) mitochondria are more stable and thus less likely to be involved in fusion/fission events, whereas longer (branched) mitochondria provide a greater surface where they can potentially form new branches or merge/fuse with other mitochondria [22, 23]. These insights at the level of whole islet β -cells continue to build on the original high resolution 3D studies of the Golgi region in the HIT-T15 cell, in which we had been able to capture some of the first convincing images demonstrating direct structural interactions between specialized membranes of the ER and mitochondria, now commonly referred to as MAM [107].

Tomography – Translating Maps into Images

With the miniaturization of probes, significant improvements to imaging instrumentation/software and the dramatic increases seen in computational power/processing speed over the past few decades, it has now become possible to map physical parameters at high fidelity as a function of position (x,y,z) or relative to the location of neighbouring significant “landmarks” within complex extended volumes and/or in a functional context. These steps require advanced imaging technologies for precise spatial sampling of the physical sample and typically result in a huge amount of data.

Most frequently, the volumes are assembled and stored as digital “stacks” of 2D pixel-thick image datasets, although the user is not restricted to viewing/analysis of the data in the same way that one would examine a set of “conventional” 2D images. Mathematically, such data are best represented in matrices for algebraic processing by fast algorithms for tomographic reconstruction of the data’s spatial density distribution in space, typically based on use of the Radon transform (http://en.wikipedia.org/wiki/Radon_transform). In this manner, we are able to visualize the physical properties of an intact sample, such as tissue, in 3D by computing an artificial 3D reconstruction.

Currently, multi-dimensional images are mathematically reconstructed from spatial and/or temporal maps of physical data in the context of many diverse experimental/image acquisition techniques, like 2D electron density maps obtained from scanning tunnelling microscopy, 2D force maps obtained from atomic force microscopy, 3D density distributions generated by axial tomography using a wide range of emitter sources/detectors (e.g. electrons, positrons, x-rays, magnetic resonance, etc.), and 3D/4D fluorescence signals reconstructed from light microscopy (e.g. two-photon microscopy, confocal laser scanning microscopy, spinning-disk microscopy, programmable array microscopy). Further, new hybrid methods that combine a number of these (e.g. confocal x-ray fluorescence, single photon emission computed tomography together with more conventional x-ray computed tomography, etc.) are constantly emerging.

Further Reading:

Beichel R, Sonka M (eds) (2006) Computer vision approaches to medical image analysis. In: Lecture notes in computer science. Springer, New York, NY

Added by the editors

Consequently, it became apparent that our expedited approach for imaging whole (insulin-secreting) cells in 3D in situ at the EM level – albeit at lower resolution – provided us with a means to define variability and offer unique insights into mitochondrial structure–function relationships within and between islet β -cells in health and disease. Moreover, the concept of mapping mitochondrial structure and function at the organelle level among islet β -cells cultured under different physiological conditions at intermediate resolution (~ 10 – 20 nm) fits extremely well with our long-term and ambitious vision of mapping out the 3D landscapes of β -cells at macromolecular resolution.

8.3.3 Mapping Interactions Between the Microtubule Cytoskeleton and Key Organelles of the Insulin Pathway at the Cellular and Nanometre Scales

Previous high resolution ET studies of the Golgi region in β -cells have already provided novel insights regarding the distribution and nature of physical interactions between microtubules and the Golgi complex [107, 167] (Fig. 8.7a–c). Among the most notable of these findings was the extent of direct association between the microtubule cytoskeleton and membranes of the *cis*-most cisterna [106] (Fig. 8.7b, c). Since new cargo is delivered to the *cis*-face of the Golgi in mammalian cells via ER-derived membrane carriers referred to either as ERGIC (ER–Golgi intermediate compartment) elements or vesicular–tubular clusters (VTCs), these observations provided further direct support for the cisternal maturation/progression model, which proposes that new Golgi cisternae are formed following the docking and fusion of these carriers at the *cis*-face of the Golgi, using the pre-existing *cis*-cisterna as a template [39, 103, 105]. In addition, microtubules within the Golgi region did not display a typical radial distribution as if organized from the centrosome, but rather they exhibited an arrangement similar to that observed in interphase epithelial cells [105, 167]. By virtue of the kinds of modifications to the process for whole cell tomography that have been outlined in the preceding figures, recent tomograms of islet β -cells reconstructed in their entirety in 3D such as the example presented in Fig. 8.2b now demonstrate sufficient image quality and resolution for microtubules to be accurately segmented at the level of whole cells in situ with high spatial fidelity. Just as for the previous high resolution glimpses afforded into the nature of physical/spatial interactions between the microtubule cytoskeleton and membranes of the Golgi complex revisited here (Fig. 8.7a–c), such data sets are currently providing an opportunity to quantitatively map microtubule interactions with all of the key compartments/organelles actively involved in insulin processing and trafficking following (pro)insulin synthesis in the ER (e.g. the Golgi complex, insulin granules and mitochondria) at high spatial precision, as well as in a complete cellular context. Like the unique insights that were recently reported by Antony and colleagues for the organization of interphase microtubules in fission yeast through a whole cell tomography approach [60], the capacity to generate comparative maps of microtubule organization from tomograms of entire islet β -cells

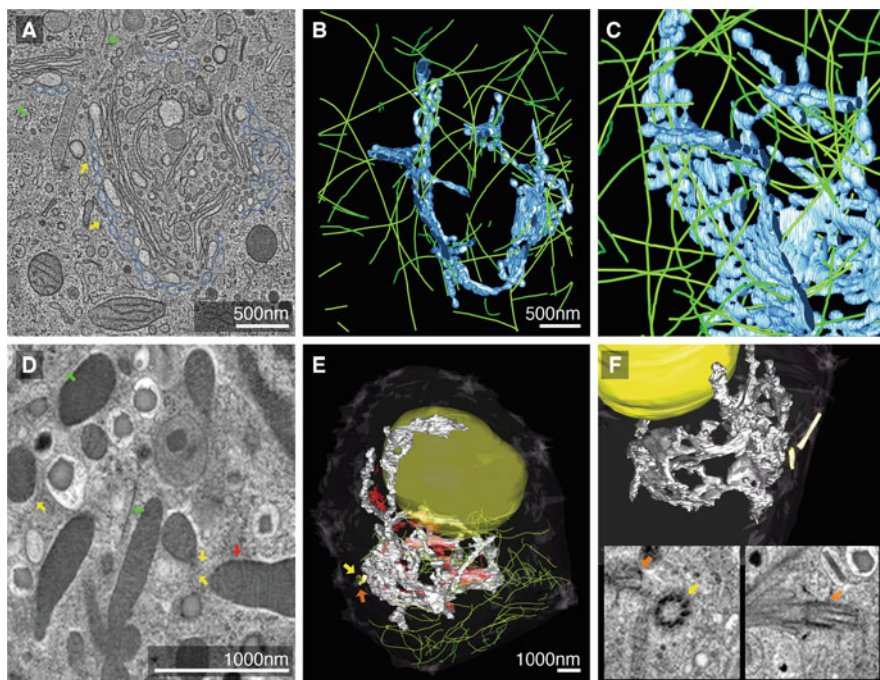


Fig. 8.7 The improved quality, accuracy and resolution of recent whole cell tomograms now affords a genuine capacity to map the microtubule cytoskeleton in situ in 3D for islet β -cells. (a) A tomographic slice from the high resolution reconstruction of the Golgi region presented in Fig. 8.1 highlights examples of microtubules oriented either parallel (*green arrows*) or oblique/perpendicular (*yellow arrows*) to the plane of the image (i.e. *XY* view), respectively. (b) A view from the 3D model derived by tracking microtubules (*bright green*) in the Golgi region, which provided new insights into the nature of interactions between microtubules and the *cis*-face of the Golgi (*light blue*), where new cargoes arrive from the ER; reproduced from data originally published in [167]. (c) A closer view reveals the striking spatial relationship between microtubules whose paths closely followed the membranes of the *cis*-most cisterna over considerable distances. Scale bars: (b and c) 500 nm. (d) An image slice from a recent whole cell tomogram of an islet β -cell (see also Figs. 8.2b and 8.3) showing the relative clarity of microtubules in different orientations for comparison with (a). (e) 3D model view showing the small subset (110) of microtubules (*bright green*) that were segmented in the vicinity of the Golgi. Colour coding: Golgi (main ribbon/*cis*-medial cisternae, *grey*; *trans*-most cisternae, *red*); nucleus (*yellow*); centrosome (*light yellow*). (f) A rotated view from the same cell that more clearly shows the position of the centrosome (*light yellow*) relative to the plasma membrane (*purple/pink*, partially transparent). The left and right insets show the daughter (*yellow arrow*) and mother (*orange arrow*) centrioles, respectively, as viewed in tomographic slices. Scale bar: 1000 nm.

reconstructed in 3D at the nanometre scale should afford exciting new advances regarding our fundamental understanding of how β -cells maintain and organize those compartments and organelles that function to process and traffic insulin, at a level of detail that cannot currently be achieved by any other imaging technique (Fig. 8.7d–f).

8.3.4 “Virtual TIRF” – New Computational Tools for Complex Quantitative Analysis of Structure–Function Variation Among Insulin Granules in Different Exocytic Pools

Quantitative studies of islet β -cell architecture at the EM level using stereological analysis of 2D images of thin sections have previously shown that β -cells in rodent islets at steady state under non-stimulated “resting” conditions typically contain between 9000 and 12000 mature insulin granules [13, 27, 129, 136, 168, 183]. Indeed, our own recent study of two different β -cells imaged following secretagogue stimulation during *in vitro* culture by exposure to elevated (11 mM) glucose for 1 hour demonstrated that “functionally equivalent” β -cells within the same islet can exhibit significant heterogeneity with respect to both mature insulin granule size and number (see also Fig. 8.1c–f). While one of the two cells appeared to have discharged the bulk of its mature granule content by the time the islet was snap frozen (i.e. the cell contained only $\sim$ 3000 mature granules), the other cell still appeared to retain close to its full complement of mature granules (i.e. $\sim$ 8000 granules) [130] (Fig. 8.8a). The original two-pool model for insulin granule exocytosis proposed by Grodsky and colleagues [55, 56] has underpinned much of our current understanding regarding the notion that different functional subsets of insulin granules provide the pipelines that supply insulin for release following an appropriate physiological stimulus (e.g. elevated extracellular glucose), largely based on spatial parameters such as proximity to the plasma membrane (Fig. 8.8b). Approximately 5% of mature insulin granules within each β -cell reside in close proximity to the plasma membrane and constitute the so-called “readily releasable pool” (RRP) of granules that are exocytosed immediately upon stimulation, thus facilitating the first phase of insulin secretion [168]. However, the majority of mature granules ($\sim$ 95%) are distributed throughout the cytoplasm and constitute the so-called “reserve pool” (RP) (Fig. 8.8b). Following stimulation, insulin granules within the RP are mobilized via both the microtubule and the actin cytoskeleton to provide an ongoing supply of granules that can sustain the longer second phase of insulin release [38, 99, 168, 198, 201]. However, although the nature of the insulin granule pool adjacent to the cell surface (i.e. the RRP) awaiting release has been extensively investigated using a variety of imaging techniques at both the LM and EM levels, the make-up of the RP remains less well defined due to the technical challenges/limitations associated with LM studies of islet β -cells as well as conventional ultrastructural studies in 2D using EM. Notably, key questions such as does the RP simply comprise the β -cell’s remaining granule population (based purely on being spatially remote from the cell surface) and/or do functionally different subsets of granules exist within the RP are still unanswered. In an attempt to address these and related questions which remain central to advancing our understanding of β -cell function, we have more recently set out to characterize the nature of subsets of mature insulin granules within these pools through the development of more robust computational approaches for analyzing complex 3D spatial data derived from cellular tomograms in a semantic context [111]. Using what we refer to as a “virtual TIRF” approach to quantitatively analyze the relative numbers, distribution and morphological characteristics of mature

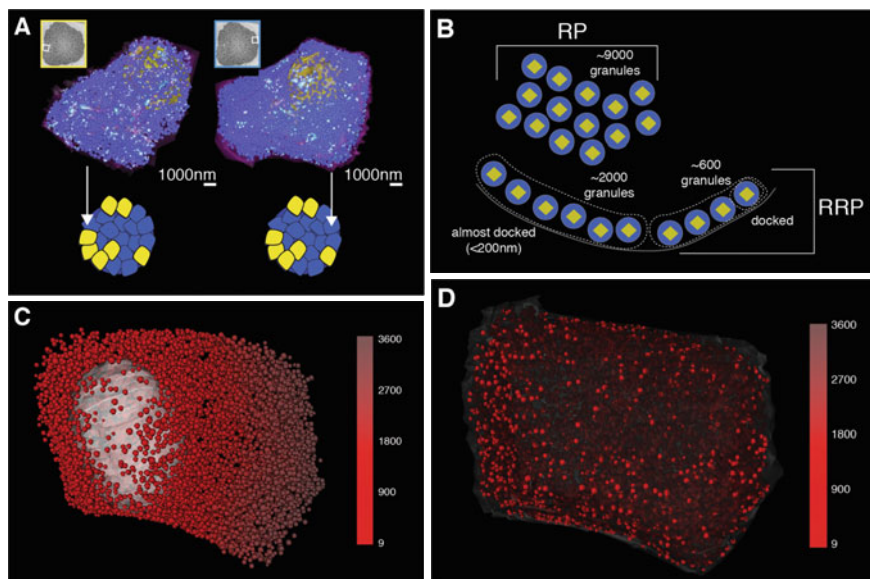


Fig. 8.8 Sophisticated computational tools now enable more complex query of β -cell organization in a semantic context using 3D spatial data modelled from whole cell tomograms. (a) Our recent ET study of two “functionally equivalent” β -cells within the same mouse islet provided direct evidence for β -cell heterogeneity with respect to size and number of the insulin granules they contained (see also Fig. 8.1c–f) and indirectly suggested that the two cells’ responses to glucose stimulation differed significantly [130]. (b) Cartoon based on the original two-pool model for insulin granule exocytosis proposed by Grodsky and colleagues [55, 56]; reproduced/adapted from Fig. 8.6B in [168]. (c) Histograms produced from precise measurement of the minimum direct (Euclidean) distance between each mature granule and its nearest point on the plasma membrane allow heat maps to be generated to aid visual inspection in 3D. (d) Both histograms and 3D heat maps can be interactively queried in a semantic context, such that any subset of granules located at any arbitrary distance from the cell surface can be visualized separately [111]. (c and d) Heat map images courtesy of Oliver Cairncross through the “Visible Cell®” project (Institute for Molecular Bioscience/ARC Centre of Excellence in Bioinformatics, The University of Queensland, Australia). Scale bars for the heat map images correspond to distance (nm).

insulin granules located at different distances from the plasma membrane, histograms produced from measurements of the minimum direct (Euclidean) distances for all insulin granules from the cell surface can be used to generate heat maps for 3D visualization (Fig. 8.8c). Since both histograms and 3D heat maps can be interactively queried in a semantic context, it thus becomes possible to visualize only a specific subset of granules (e.g. the RRP) based on their physical proximity to the plasma membrane (Fig. 8.8d). Most importantly, the 3D spatial coordinates of different granule subsets generated from each query can then be exported as sets of data points which can be viewed in the context of the original 3D image volume (since the 3D model points correspond to the centroids of the granules that were originally

segmented within the whole cell tomogram), allowing further structural interrogation of granule morphology/morphometrics within the primary tomographic volume (not shown).

8.4 Conclusions and Future Directions

Our whole cell tomography approach that yields 3D cellular maps at “intermediate resolution” now affords us a practical method for determining precisely how mitochondrial structure–function relationships change at both the intra- and the inter-cellular level in islet β -cells imaged in different physiological states. Quantitative data obtained in this manner for multiple β -cells will both inform and be informed by the information generated in parallel from high resolution 3D studies of β -cell organization undertaken for large cellular subvolumes. As demonstrated in Figs. 8.6, 8.7 and 8.8, our expedited approach for studying β -cell biology in situ in 3D now affords precise quantitative data to be obtained for mitochondrial organization, insulin granule subsets within the larger granule pools as well as for mapping the microtubule cytoskeleton at ~ 10 nm resolution at the cellular scale. Further, this approach is both temporally and fiscally practical for generating sets of 3D organelle maps for different islet β -cells imaged following culture in vitro under different conditions that reflect key physiological states relevant to normal physiology, as well as environmental conditions that lead to β -cell dysfunction and/or death [93, 96, 189].

There is now a clear desire/need within the international islet/ β -cell biology, diabetes and biomedical research communities to understand the “bigger picture” with respect to the β -cell as complex systems, and using a precise spatial framework as a “foundation” is considered by many in systems biology as a fundamental prerequisite to developing a genuine capacity to accurately model changes in the spatio-temporal coordinates of complex cellular events in silico and ultimately for predicting the pathophysiology of chronic diseases like diabetes and cancer in the future [14, 20, 93, 161]. By combining new mathematical and computational tools for precisely extracting useful, novel and quantitative insights into 3D cellular organization from these enormous data sets, we foresee a unique opportunity to begin mapping structural phenotypes for β -cell function versus dysfunction onto complete sets of 3D spatial coordinates for β -cell organization with nanometre precision.

Supplementary Online Material

Multimedia files generated as *QuickTime*® movies from sets of TIF/PNG images captured directly from tomographic data using the *3dmod* viewer in the *IMOD* software suite are provided as online supplemental data to accompany several of the figure panels presented here as sets of static images.

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Chapter 9

In Vivo Applications of Inorganic Nanoparticles

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Abstract Chapter 9 is primarily concerned with in vivo applications of nanoparticles. This very broad review includes aspects such as bioconjugation, which is a pre-requisite for any in vivo application, and nanotoxicity. We introduce the two main fields of in vivo applications of nanoparticles: bioimaging and therapy. In the field of imaging, magnetic resonance imaging and optical imaging are distinguished, and the latter is further subdivided into groups of luminophores. These groups include gold nanoparticles, semiconductor quantum dots and rare-earth-doped nanoparticles. In Section 9.4, we discuss the methods of hyperthermia, photodynamic therapy and magnetic targeting. The aim of this chapter is not to provide in-depth insights into the different applications but to give an overview of possibilities and limitations when nanoparticles are used within living organisms.

Keywords Imaging · In vivo · Nanoparticles · Therapy · Toxicity

Abbreviations

AC	Alternating current
AuNP	Gold nanoparticle
AuNR	Gold nanorod
BSA	Bovine serum albumin
DHLA	Dihydrolipoic acid
DNA	Deoxyribonucleic acid
DTPA	Diethylenetriaminepentaacetic acid
DTT	Dithiothreitol
EDC	1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide
FRET	Förster or fluorescence resonance energy transfer
GPC	Glial progenitor cell
Hb	Haemoglobin

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HbO ₂	Oxyhaemoglobin
HDA	Hexadecylamine
HFMF	High-frequency magnetic field
IR	Infrared
MEMRI	Manganese-enhanced magnetic resonance imaging
MRI	Magnetic resonance imaging
MTH	Mild-temperature hyperthermia
NIR	Near-infrared
OCT	Optical coherence tomography
PDT	Photodynamic therapy
PEG	Polyethylene glycol
PPTT	Photoacoustic imaging and plasmonic photothermal therapy
QD	Quantum dot
RES	Reticular endothelial system
ROS	Reactive oxygen species
SAR	Specific absorption rate
ScFv	Single-chain variable fragment
SMCC	4-(<i>N</i> -Maleimidomethyl)-cyclohexanecarboxylic acid <i>N</i> -hydroxysuccinimide ester
SPION	Superparamagnetic iron oxide nanoparticle
SPR	Surface plasmon resonance
TOPO	Trioctylphosphine oxide
TPL	Two-photon luminescence
UCN	Upconverting nanocrystal
UV	Ultraviolet

9.1 Introduction

The development of functional inorganic nanoparticles (NPs) has progressed exponentially over the past two decades. Examples from the diverse range of available NPs include magnetic nanocrystals [1], luminescent particles [2] and sophisticated systems such as upconverting NPs [3]. This “toolbox” of available functionalities enables the realisation of different *in vivo* diagnostic and therapeutic applications. There are many factors to be taken into account when using inorganic nanocrystals as reporters or drug delivery systems. Some issues are generic such as nanotoxicity but others are specific to the type of particle. In this chapter, we will discuss the most important topics related to the *in vivo* application of inorganic nanocrystals.

The synthesis of nanocrystals represents a major challenge in nanotechnology. Since nanocrystal synthesis does not fall under the remit of this chapter, we will refer to recent articles on this issue. The most common type of magnetic NP used is magnetite (Fe₃O₄), which is typically prepared using a thermal reaction between iron complexes with carboxylic acids and/or alcohols [4, 5]. The vast majority of

luminescent semiconductor nanocrystals, so-called quantum dots (QDs), consist of cadmium selenide (CdSe) [2]. However, cadmium-free alternatives such as indium phosphide (InP) have recently come to the fore [6–8]. Other luminescent NPs such as luminescent gold [9] or carbon are relegated to a niche existence [10]. Similarly, the field of rare-earth-doped nanocrystals is dominated by upconverting NPs. The most successful systems in this area are lanthanide phosphate- [11, 12] and sodium yttrium tetrafluoride-based nanocrystals [3, 13, 14]. The synthesis of gold NPs (AuNPs) is well established and has altered very little over the last century [15–17].

The key problems in the exploitation of nanocrystals in the life sciences are to stabilise the colloidal NPs in high-ionic-strength aqueous buffers and to couple targeting moieties or biomolecules to their surfaces. Section 9.2 is concerned with the conjugation of NPs to biomolecules. This involves an initial ligand exchange step and may require very different procedures depending on the nature of the core nanocrystal.

Section 9.3 is concerned with imaging applications where the vast majority of nanocrystals are used as contrast agents or luminophores. Magnetic NP dispersions for use as magnetic resonance imaging (MRI) contrast agents are at present commercially available. Moreover, a whole chapter in this book is dedicated to MRI. Therefore, we will concentrate on the problems and recent developments in this area which are specific for the application of NPs. There are criteria which need to be met in order for a potential fluorescent label to be suitable in biological systems. The excitation of the label and the biological matrix should be mutually exclusive as far as possible. The fluorescence should be bright as to be detected with conventional instrumentation (i.e. possesses a high molar absorption coefficient at the excitation wavelength and a high fluorescence quantum yield). The probe needs to be soluble in buffers that are relevant to cell culture media or body fluids. Also, the probe must be sufficiently stable under relevant conditions and be available in a reproducible quality. Additionally, it should have functional groups for site-specific labelling and reported photophysics. Specific considerations for in vivo imaging include steric- and size-related effects, the possibility to deliver the label into cells, NP cytotoxicity, multiplexing suitability and other practical issues. These demanding pre-requisites are difficult to meet by any label; however, we will discuss the possibilities of fluorescent labelling with QDs and other luminescent NPs.

Therapy using inorganic NPs can be subdivided into two major groups: utilisation of magnetic NPs and luminescent nanocrystals. It should be noted that drug delivery by exploitation of nano-capsules does not fall under the remit of this chapter since these capsules are usually made of organic material. Magnetic NPs can be used for magnetic targeting and magnetic hyperthermia, whilst luminescent NPs are mostly used for the creation of reactive oxygen species (ROS), Section 9.4.

Toxicity is a much discussed but little studied topic where nanocrystals are concerned. Nevertheless, since information on the toxicity of NPs is crucial for any in vivo application, we will finish this chapter by reviewing the available literature on this important topic. Further information can be found in Chapter 18.

9.2 Bioconjugation

Nanoparticles (NPs) of different sizes, shapes and compositions are a promising avenue for biomedical research due to their unique characteristics [18]. Advances in this field have been slow due to the limitations encountered in decorating inorganic NPs with appropriate functional groups. Functionalisation is used to make the NPs soluble and stable in aqueous media. This increases biocompatibility and biofunctionality while preserving their original properties [19].

Most of the synthetic routes employed to obtain NPs are carried out in organic, non-polar solvents. This gives rise to nanocrystals stabilised with coordinating ligands and thus the NPs are insoluble in aqueous media. Common examples of stabilising ligands include trioctylphosphine oxide (TOPO), hexadecylamine (HDA) and oleic acid (OA). On the other hand, there are routes for the synthesis of NPs that are performed in aqueous solution but lead generally to a loss of NP quality. In the case of quantum dots (QDs), they have the drawback of generating NPs of poor quality in terms of monodispersity, crystallinity or fluorescence efficiency. Nevertheless, citrate-stabilised gold nanoparticles (AuNPs) synthesised in aqueous solution produce high-quality nanocrystals. Post-synthesis surface ligand exchange of all of the previously mentioned NPs is required to impart improved colloidal stability in aqueous systems (hydrophilicity) and the desired functionalisation to the NPs for biomedical applications (see Fig. 9.1). According to the literature, there are two approaches to phase transfer that convey water solubility to NPs: ligand exchange and amphiphilic polymer coating [20].

The ligand exchange strategy, firstly described by Chan and Nie [21] and Alivisatos et al. [22], is based on the replacement of the original hydrophobic ligands adsorbed onto the surface of the nanocrystal with bifunctional molecules. These molecules possess one end with a functional group with a higher affinity towards the nanocrystal surface than the original ligand which drives the exchange. For instance, a thiol group ($-SH$) will displace a carboxyl group ($-COOH$) acting as a stronger “anchoring” group. The other end of the new ligand can yield the required functionality to the NP. For example, a ligand with an aliphatic hydrocarbon chain ($-C_nH_{2n+1}$) can be exchanged for a carboxyl ($-COOH$) or a sulphonic ($-SO_3H$) group. At neutral or basic pH, carboxyl and sulphonic groups are deprotonated, and the negative charge of the nanocrystals produces electrostatic repulsion between the NPs, thus avoiding particle aggregation [23]. However, this strategy also presents some important limitations such as the lack of long-term stability in biological buffers. This is a consequence of the labile bond between the NP surface and the thiolated ligands, which desorbs from the surface over time leading to aggregation and precipitation of the NPs [24]. In order to achieve increased aqueous stability, bidentate ligands such as dithiothreitol (DTT) and dihydrolipoic acid (DHLA) modified with poly(ethylene glycol) (PEG) can be used to generate more stable coatings compared to those based on monodentate ligands due to the presence of multiple anchoring points [25, 26].

A family of compounds that are used frequently to substitute the hydrophobic ligands are silanes, which act as silica precursors, such as

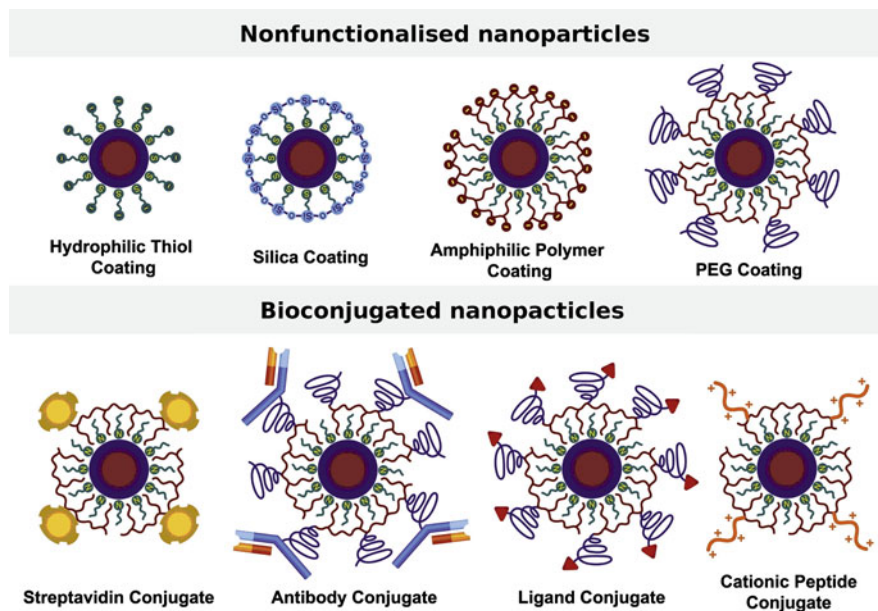
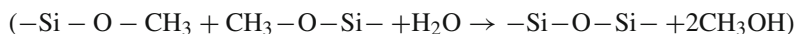


Fig. 9.1 Scheme of the most relevant strategies to modify the surface of the NPs: (*above*) to render NPs soluble in aqueous environments; (*below*) to address biological functionality to the NPs. Reprinted from *Advanced Drug Delivery Reviews*, 60/17, A. M. Smith, H. Duan, A. M. Mohs and S. Nie, *Bioconjugated quantum dots for in vivo molecular and cellular imaging* from Elsevier.

3-mercaptopropyltrimethoxysilane (MPS). The thiol group reacts with the surface of the NPs and the methoxysilane groups ($-\text{Si}-\text{O}-\text{CH}_3$) can react with each other to form siloxane bonds:



In the second step, silane monomers containing functional groups such as PEG, ammonium or carboxylate can be added to grow a thicker and more stable inorganic shell, as well as to impart the desired functionality for further bioconjugation [27]. In this way, a highly cross-linked and stable silica shell is deposited around the surface of the nanocrystal [28]. This approach, even though its principle is based on surface ligand exchange, has generated a significant number of publications. Some authors consider it a third strategy called silica coating or surface silanisation [29]. The creation of a thick silica shell around NPs is well established but the overall size of the NPs is a limiting factor for numerous applications. Establishing a general protocol in order to acquire thin silica coatings that work in different types of NPs is a current challenge for the nanotechnology community.

A second strategy to fabricate water-soluble NPs is the use of an amphiphilic polymer. An amphiphilic polymer contains polar and non-polar subunits which

allow the hydrophobic chain to have sides which have opposite polarities. The binding of the polymer to the hydrophobically ligated NP is based on the interaction of hydrophobic alkyl chains of the polymer with the alkyl chains of surface ligands, forming a bilayer. Consequently, the hydrophilic groups are located on the exterior part of the shell rendering water solubility to the NPs [30–33]. This procedure retains the native surface ligands from the synthesis of the nanocrystal. Therefore many of their photophysical properties are not modified but the size of the NPs is much larger than that of those coated with a monolayer of ligand [30]. In fact, the diameter is increased from three to four times larger than the original particles, which is a disadvantage for several in vivo applications [31].

In addition to those general strategies, the recent literature has described several methods to transfer different types of NPs into aqueous media such as phospholipid-encapsulating layers [34].

The now hydrophilic NPs can be conjugated to biological probes such as nucleic acids, proteins and small molecules for FRET-based sensors (Förster or fluorescence resonance energy transfer). Currently, there is no universal conjugation scheme that can be used to attach different biological species to the NP surface. The coupling can be via adsorption, electrostatic interaction, covalent linkage or by using biological interactions such as biotin–avidin [35].

If the biological molecule possesses functional groups that are reactive towards the NP surface, such as thiols, they can easily interact with the nanocrystal surface replacing some of the original ligands that stabilise the NPs. This bioconjugation model, which is based on adsorption, has been demonstrated to obtain gold NP bioconjugates with molecules such as oligonucleotides, peptides or PEG [36, 37].

The carboxyl group is one of the prevalent functionalities that NPs possess after the water solubilisation process. The aforementioned functionality gives the nanocrystal surface a negative charge when dispersed in neutral or basic buffers. Negative surface charge allows direct self-assembly with positively charged biomolecules conjugated through electrostatic interactions. This scheme has been followed to attach cationic avidin proteins or recombinant maltose-binding proteins fused with positively charged peptides to negatively charged NPs [38]. It is noteworthy that due to the high surface curvature of the NPs, there is a reduction in the steric hindrance related to the packing of the biomolecules. Therefore, it is possible to increase the density of surface molecules per unit area on nanocrystal surfaces relative to the bulk material [39]. This has allowed the creation of NPs bioconjugated with mixed-protein surfaces, where each protein imparts a different functionality [40].

Classic bioconjugate chemistry is employed frequently to attach biomolecules to the NPs by covalent linkage. Cross-linkers such as EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) are used to link the terminal carboxylic groups from the NPs coating with amino groups on the biological molecules. Alternatively, SMCC (4-(*N*-maleimidomethyl)-cyclohexanecarboxylic acid *N*-hydroxysuccinimide ester) is used to bind amino-functionalised NPs with exposed thiol groups. This has been reported in several publications including conjugation to biotin [41], peptides [42], aptamers [43], avidin/streptavidin [44] and

antibodies [45]. A drawback to this approach is the risk of cross-linking between particles when the biomolecule presents several reactive functional groups. This is found commonly in big molecules such as antibodies [20]. The coupling of polyhistidine tags to carboxylated NPs can also be achieved using this method. Addition of a simple polyhistidine tag to the biomolecule of interest allows for the bioconjugation of the NP [46].

The avidin–biotin system has been widely used in biomedical research, and it is well known that the tetramer avidin interacts stoichiometrically with biotin. The high affinity of biotin ($K_d \sim 10^{-15}$ M) allows the binding of one biotin molecule per subunit. The conjugation of NPs with avidin, streptavidin or neutravidin via electrostatic interaction or covalent linkage is usually employed as basic building blocks for further bioconjugation. By mixing tetramer-decorated NPs with biotinylated molecules, the desired bioconjugate can be prepared quickly and with relative ease [47].

PEG-coated NPs are hydrophilic and biologically inert due to the PEG layer optimally masking the particles. PEG introduces steric bulk to the NP surface, which causes the particles to repel each other, increasing colloidal stability. Moreover, PEG reduces the non-specific adsorption in cells and decreases the rate of clearance from the bloodstream after intravenous injection [20].

Optimisation of surface conjugation of biomolecules to NPs is an area of current research. Remaining requirements for satisfactory bioconjugation are the preservation of NP properties such as colloidal stability, size and physical properties, which may include fluorescence or magnetism. The functionality of the biomolecule–NP conjugate is as important as the attributes of the NPs. It has been found that in some cases, it is difficult to maintain the biomolecule's functionality or its binding strength [48].

9.3 Imaging

Nanoparticles (NPs) are within the same size domain as many biomaterials including enzymes, antibodies and protein receptors. This combined with the unique properties of materials in the nano-size range provides scope for making measurements more efficiently than existing molecular materials. Prominent properties include the high quantum yields for fluorescence and large magnetic moments found in certain types of particle. NPs conjugated to biomolecules can exploit the specificity of the biomolecule to supply biotargeting functionality to the NP [46]. This makes NPs ideal candidates for in vivo imaging applications. This section provides an overview of current uses of NP in in vivo imaging.

9.3.1 Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is based heavily on nuclear magnetic resonance (NMR) and was first patented by Damadian (1972), culminating in the

first MRI image in 1978 [49]. A detailed explanation of MRI is provided earlier in this publication and by Högemann and Basilion [50] and Mitchell and Cohen [51].

A “contrast agent” is often employed to enhance an MRI image, giving a sharper contrast between soft and hard tissues in the body [52]. Traditional relaxation agents have been based on paramagnetic gadolinium chelates, such as diethylenetriamine-pentaacetic acid (DTPA), but these have their limitations. Indeed, nanostructured systems [53] have been synthesised in order to deliver large payloads of gadolinium chelates to a targeted area, but their large size (over 100 nm) can lead to in vivo rejection by the reticular endothelial system (RES) [54]. MEMRI (manganese-enhanced MRI), another well-documented technique, has unique contrast properties but manganate (Mn^{2+}) ions, like gadolinium ions, have toxic effects [55].

Magnetic NPs, particularly iron oxides, maghemite (Fe_2O_3) and magnetite (Fe_3O_4), are of great interest and are used widely in the field of MRI due to their superparamagnetism [56]. These superparamagnetic properties allow for the facile alignment of the aforementioned particles’ magnetic moments to an applied magnetic field, making them ideal contrast agents. Nanoparticulate contrast agents preferentially have high magnetisation values, a diameter smaller than 100 nm and a narrow particle size distribution in order to be suitable for in vivo applications [52]. Despite good relaxation properties, the negative contrasting effects of iron oxide could potentially lead to clinical misdiagnosis [53].

Superior contrasting effects in comparison to Fe_xO_y are possible by using different types of magnetic NPs with greater magnetic moments [57]. It has been found that by altering the metal “M” in MFe_2O_4 NPs, the magnetic moment can change dramatically, with the highest magnetic moments giving the best agents [58]. Among the finest nanoparticulate contrast agents with a large magnetic moment are MnFe_2O_4 nanocrystals. It has been reported that MnFe_2O_4 nanocrystals with an antibody conjugate (Herceptin) which target cancerous cells effectively has been used successfully for in vivo MRI in mice [57].

Gold nanocomposites have been used as excellent MRI contrast agents in vivo, giving a superior contrast compared to traditional agents [57]. State-of-the-art contrast agents involve hybrid NPs [60]. Examples include a superparamagnetic magnetite core with a layer of silica before gold encapsulation and the poly(ethylene glycol) (PEG) coating of magnetite/gold NPs to ensure biocompatibility [61].

Rare-earth inorganic NPs and nanocomposites represent the next generation of MRI contrast agents. Gd_2O_3 , GdPO_4 and GdF_3 are excellent nanoparticulate candidates for in vivo MRI imaging. However, the inherent toxicity of gadolinium and other rare-earth elements coupled with the difficulty in synthesising monodisperse examples and non-facile surface protection has led to limited in vivo applications thus far [62]. Functionalised manganese oxide (MnO) NPs have been used as extremely effective, biocompatible contrast agents in vivo [63]. Recently, lanthanide complexes have been grafted onto silver NPs and are under development as advanced, biocompatible, composite MRI contrast agents [64].

NPs have been used successfully and extensively in vivo for MRI and are continually refined and improved with more novel and innovative composites, such as FeCo particles with graphitic shells for MRI [65]. Together, MRI and nanoscience represent a bright future for in vivo imaging, especially when used in tandem with optical imaging techniques for diagnosing diseases such as cancer.

Inorganic Nanoparticles

Labelling Properties in Structures

All physical methods suffer from limited resolution. This means a property measured by a method can only be attributed to a location with some precision that cannot, for the moment being, be further refined. In optical microscopy, this limit is on the order of ~ 200 nm, and for electron microscopy, it is around ~ 1 Å. All details beyond these limitations still contribute to the image assembly but cannot be resolved; if magnified further, the image appears to be blurred.

One way out is use of specific labels that bind to a substructure that cannot be visualised directly. Still, the chemical specificity of the label, if carefully selected, allows for the detection of internal features that cannot be imaged in detail. The first labels that were used in optical microscopy were simple dyes that modified the absorption properties within a sample and thus entered detectable marks into it. In the next generation, fluorescent dyes were used that gave, at even very low concentration, rise to brilliant signals. Thus the modification of the sample properties due to the presence of the label is reduced, still at detectable signal levels. The green fluorescent protein (GFP) is a prominent example for that kind of label as its synthesis can be encoded into the protein factory of many cells (Tsien R (1998) The green fluorescent protein. *Ann Rev Biochem* 67:509–544).

Alternatively, small (nanometre size) particles could be used to label molecules by being chemically linked to them. The first nanoparticles to be used for that purpose were gold particles. This kind of marker is giant in size as compared to the structure that it is labelling. Usually, gold particles can be directly detected because they induce dark spots into images (in optical and in electron microscopy). This makes them suitable for particle tracking methods in optical microscopy. Moreover, the presence of metallic/gold nanoparticles may serve to enhance Raman resonance affinity and intensity. Thus, spatially resolved spectroscopic data may be added to the pure detection of the labelled molecules or the tracking of their mobility.

Most recently, nano-diamonds were synthesised that will accomplish the quantum dots (Yu S-J et al (2005) Bright fluorescent nanodiamonds: no photobleaching and low cytotoxicity. *J Am Chem Soc* 127(50):17604–17605).

Further Reading:

Vo-Dinh T (ed) (2007) Nanotechnology in biology and medicine: methods, devices, and applications. CRC Press, Boca Raton, FL

Added by the editors

9.3.2 Optical Imaging

The field of biological optical imaging is undergoing rapid development. This is a consequence of the continuous development in materials science, physics and chemistry [66]. This section provides an insight into state-of-the-art NP optical imaging techniques, emphasising the different types of NPs involved. The NPs focussed upon in this section, such as quantum dots (QDs), gold (AuNPs), and rare-earth doped NPs, are those which have shown the greatest potential for mainstream medical imaging.

9.3.2.1 Gold Nanoparticles

The unique optical properties of colloidal metal NPs have been used for centuries. In ancient Egypt, colloidal gold particles were used to decorate pottery due to their characteristic ruby-red colour. It was not until 1857 when Faraday performed the first scientific investigation into the ruby-red colour of the, at the time unknown, gold-containing material, where he attributed the colour to the colloidal nature of the particles [67]. In 1908, the visible absorption profile was explained using Maxwell's electromagnetic equations by Mie [68].

The interaction of light and AuNPs is the principle of the detection techniques described in this chapter, which are generally based upon harnessing the surface plasmon resonance (SPR). A surface plasmon is an excitation band generated by the collected excitations of conduction electrons [69]. In a similar manner to QD fluorescence, the SPR is dependent on the size of the AuNP and undergoes a red shift with increasing size [70]. Moreover, near the surface plasmon the absorption cross section becomes extremely high when compared to organic dyes. It should be highlighted that AuNPs of non-spherical shapes, such as gold nanorods (AuNRs), may have multiple plasmons which are non-degenerate due to the removal of symmetry within the particle [70]. Thus, AuNRs will possess two surface plasmon bands, one due to oscillations along the length (longitudinal band) and another from oscillations across the width of the rod (transverse band) [71]. The length of AuNRs can be controlled and the position of the transverse band can be tuned between 650 and 1000 nm making the AuNRs very useful in optical bioimaging [72]. Gold nanoshells are a third type of gold NP in this chapter, which consist of a silica particle at the core with a gold shell of various thicknesses. The core/shell size ratio controls the position of the SPR which allows the tuning of the SPR for bioimaging [73].

Utilisation of the surface plasmon and the large extinction coefficient underpins the optical techniques used *in vivo*. It is worth paying attention to a large restraint in the development of *in vivo* optical imaging techniques. The window for minimal light absorbance of H₂O, oxyhaemoglobin (HbO₂) and haemoglobin (Hb) falls in the near-infrared (NIR) between 880 and 660 nm (Fig. 9.2) [74]. This constraint hinders the use of optical imaging at great depths within a subject.

This chapter will provide an introduction to the *in vivo* optical imaging of AuNPs and an overview of the techniques employed for such a purpose. A more general use

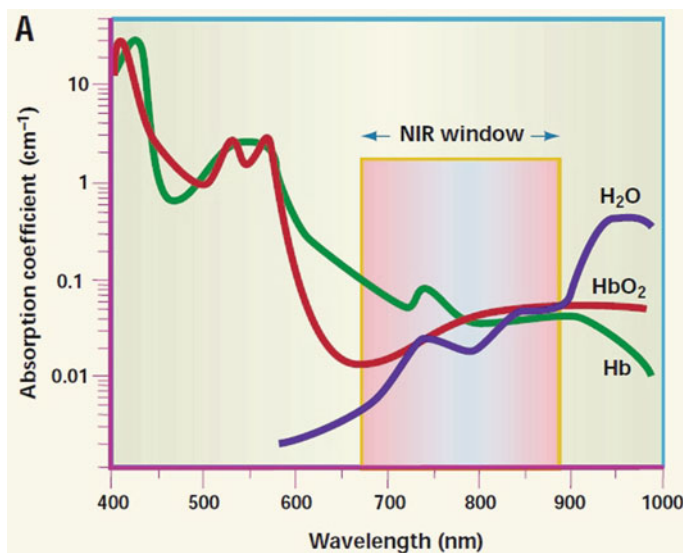


Fig. 9.2 The near-IR window that optical imaging uses for maximum efficiency. Reprinted by permission from Macmillan Publishers Ltd: [Nature Biotechnology] 19(4):316–317, copyright (2001).

of AuNPs in bionanotechnology has been reviewed recently by Sperling et al. and Boisselier and Astruc [36, 75].

Optical coherence tomography (OCT) has recently used the high scattering ability of gold nanoshells as a contrast agent *in vivo* [76]. OCT has a micrometre-scale resolution and provides a two-dimensional subsurface image. In OCT, a fibre-optic Michelson interferometer is illuminated by low coherence light (830 nm). The sample is placed on one arm of the interferometer and a reference mirror on the other. The reflected beams recombine, creating interference patterns which allow an image to be created [77]. The extra scattering achieved using a gold nanoshell increases the contrast of the image, and this property has been used to increase the contrast of tumours in mice. The gold nanoshells accumulated preferentially in the tumour and increased the contrast more than in other tissue [76].

Photoacoustic imaging and plasmonic photothermal therapy (PPTT) are two different techniques that take advantage of the selective absorbance of the surface plasmon resonance and the fact that the NPs relax by releasing heat into their surrounding environment [78, 79]. The main difference is that in photoacoustic imaging a pulse of NIR laser light, typically 757 nm, is used in resonance with the surface plasmon instead of a continuous NIR source. Such a pulse of NIR light causes rapid thermal expansion of the surrounding media and the generation of a sound wave that can be detected on the surface of the subject. The use of NIR reduces the amount of absorption that occurs by the light, but absorption of the light by various other organs is unavoidable. Therefore, the resulting optoacoustic signals have contributions generated by the organs of the subject and a distinct signal from the gold NPs.

PEG-coated gold nanoshells have been used as a contrast agent to image their distribution circulating in the vasculature in rat brain using photoacoustic imaging [80]. Due to the ability of AuNRs to have the maximum of the plasmon resonance tuned further into the NIR, *in vivo* images have been collected using a mouse as a subject [81]. The lymph system of a rat has been imaged by using AuNRs and photoacoustic imaging [82].

In two-photon luminescence (TPL) spectroscopy, an electron is excited from the conductance band to the valance band of the AuNPs using two photons. As the electron relaxes to the conductance band, light is released as fluorescence, which can be used for imaging. Non-linear processes involving two photons for the excitation are weak due to their low probability of occurrence. TPL harnesses the fact that the surface plasmon resonance of metallic NPs is known to amplify a variety of linear and non-linear optical properties, such as two-photon excitations [83]. The weak electronic transitions couple to the surface plasmon allowing the probability of two-photon excitation occurring to increase [84], and the resulting relaxation in the form of luminescence can be collected to form a three-dimensional image. This approach has been demonstrated by Wang et al. [85] where they have collected images of single AuNRs flowing in mouse ear blood vessels with luminescence three times stronger than the background luminescence.

It is also known that upon adsorbing a molecule to the surface of a colloidal AuNPs, the Raman scattering efficiencies of the molecule are amplified as much as 10^{14} - to 10^{15} -fold [86]. Recently this fact has been exploited *in vivo* to act as a finger print to show the location of AuNPs [87]. The adsorbed organic dye molecule, known as Raman reporter, was not displaced when a thiol-modified PEG was exchanged with the stabilising ligands to provide water solubility and biocompatibility. As the emission and excitation spectra of the bioconjugate were in the near-IR window, the particles were over 200 times brighter than the tested QDs. Finally, the resulting particles were further functionalised with the ScFv (single-chain variable fragment) antibody to allow active targeting of epidermal growth factor receptors on human cancer cells.

Although there are few published examples of the use of AuNPs *in vivo*, including AuNRs and gold nanoshells, a considerable amount of studies have been reported in the literature where imaging has been tested *in vitro* and its potential for future *in vivo* studies is shown [36, 76, 88].

9.3.2.2 Quantum Dots

Colloidal quantum dots (QDs) are inorganic semiconductor nanocrystals of a few nanometres in diameter with unique optical and chemical properties, but complicated surface chemistry [89]. QD size and shape can be precisely controlled by adjusting the duration, the temperature and the ligand molecules used in the synthesis [90]. The size and tunable properties of these NPs are one of the features that has made them very attractive tools in biology, therapeutics and other life sciences [91–95].

QDs have been proposed as substitutes for traditional organic dyes and fluorescent proteins in optical imaging due to their unique photophysical properties. For instance, QDs are about 10–100 times brighter and show narrower and more symmetric emission spectra than are organic fluorophores. Furthermore, they present large absorption cross sections, which make them 100–1000 times more stable against photobleaching. A single light source can be employed to excite several QDs with different emission wavelengths spanning the electromagnetic spectrum, from the ultraviolet to the NIR [21, 22, 66, 95].

In the last decade, water-soluble bioconjugated QDs have been employed increasingly for cell labelling and imaging [21, 22]. Other techniques for the preparation of biocompatible QDs were being developed in tandem [96, 97]. These developments have allowed cell targeting using QDs bioconjugated to antibodies and peptides [34, 98–101]. Nevertheless, intracellular targeting of QDs has been found to be more complicated when compared to extracellular targeting, as different factors could affect the uptake of the QDs to cells. One of the most important properties of the final bioconjugate QD–biomolecule is the overall size. This is because a large size will have an adverse effect with regard to membrane protein trafficking and minimises the accessibility of populous locations within the cells, cell viability, cell size and cellular diversity [102–104].

A variety of techniques have been extensively used for intracellular labelling with QDs, for example passive uptake, receptor-mediated and non-specific endocytosis, cell penetration, liposome-mediated intracellular delivery, electroporation and microinjection [34]. In particular, the last two are widely used in transformation and transfection of recombinant genetic materials to prokaryotic and eukaryotic cells [102, 105]. Electroporation of QDs into cells is based on the application of electric pulses, which temporarily disturb the phospholipid bilayer, thus increasing permeability of cellular membranes. This has been applied successfully in cell tracking and cytometry. However, it may cause aggregation of QDs inside the cells, leading to cell death. On the other hand, microinjection is a straightforward mechanical technique in which QD conjugates are introduced homogeneously into the cytoplasm or the nucleus of the cell by applying a pneumatic pressure or an electrical impulse. Nevertheless, the main drawback of this method is the need to manipulate individual cells carefully, making the preparation of large amounts of samples more convoluted [20].

Cellular uptake of QDs is a very important factor when discussing in vivo applications of NPs. For instance, Biju et al. (2007) employed streptavidin-coated QDs functionalised with the neuropeptide allatostatin, a known transfection agent for human epidermoid carcinoma cells. As a consequence, cytoplasm and nuclear uptakes of the QDs were investigated [103]. In other applications, CdSe/ZnS QDs were encapsulated in phospholipid block copolymer micelles and delivered into *Xenopus* embryos by microinjection, allowing for the study of embryogenesis dynamics [34]. Glial progenitor cells (GPCs) over-express the platelet-derived growth factor and its receptor, playing an important role in the development and growth of glioma, and affecting multiple biological processes. In this sense, streptavidin-coated QDs have been conjugated to biotinylated antibodies and

delivered successfully into GPCs by liposome-mediated delivery. This potentially opens a door to future mechanistic studies of oncogenic signalling events in real time [106].

Even though QDs have been employed successfully for *in vitro* applications on numerous occasions [24, 107, 108], the development of QD probes for imaging inside living cells is still challenging. This is due primarily to the lack of robust methods for delivering monodispersed QDs into the cytoplasm of living cells. QDs tend to aggregate inside cells and are most likely immobilised in endocytotic vesicles such as endosomes and lysosomes. This is a significant drawback that needs to be overcome by optimising size, shape and surface coating of the imaged QDs [20].

In vivo tracking of cells is an area of research in which QDs are particularly suited, as they can be easily uptaken by cells *in vitro*. *In vitro* loading of cells before *in vivo* application was first demonstrated by micro-injecting QDs into cytoplasm of single frog embryos, where luminescence was retained throughout cell growth [34]. Hoshino et al. [109] and Voura et al. [110] injected mice intravenously with bioconjugated QDs and tracked their uptake into internal organs by monitoring the *in vivo* fluorescence once dissected. Gao et al. [111] loaded QDs into human cancer cells *in vitro* and the *in vivo* application of the cells in mice was tested. The human cancer cells divided and formed a tumour which could be visualised using fluorescence. Fluorescent labelling of multiple areas in the body with high specificity is achievable with targeting and QDs of differing sizes, leading to advanced NP probes for *in vivo* tracking [112–114].

QDs have shown tremendous promise when imaging the vascular networks of mammals such as lymphatic and cardiovascular systems. In 2004, Kim et al. [112] demonstrated that the NIR fluorescence of QDs could be used to locate the position of sentinel lymph nodes in mice and pigs. The migration of QDs within the lymph system and the localisation in nodes have been further observed by Soltesz et al. [115]. Examples of the movement of QDs through the lymph system to nodes show great potential in aiding surgeons to locate and remove lymph nodes when needed [116, 117]. Furthermore, the multiplexing abilities of QDs can be used to map lymph drainage networks. Multiplexing takes advantage of the ability to tune the luminescence colour with particle size but the excitation band remains the same. In the case of optical bioimaging, QDs that target different areas can be chosen to be of different colours but are excited simultaneously. Injection of QDs at different locations intradermally to observe the drainage to multiple nodes in real time [118] or to a common node [119] allows the mapping of the lymph network (Fig. 9.3).

The imaging of cardiovascular systems has also been achieved using QDs. In 2003, it was demonstrated that after injection, QDs retained their fluorescence and were detectable in the capillaries of skin and adipose tissue of a mouse [120]. Other NIR-emitting QDs have been used to image the coronary vasculature of a rat heart [121] and the blood vesicles of chicken embryos [122]. It was also shown that QDs have a greater sensitivity than traditional molecular imaging probes [122]. The ability to use multiplexing techniques to image the cardiovascular system shows similar potential to that observed when imaging the lymph system.

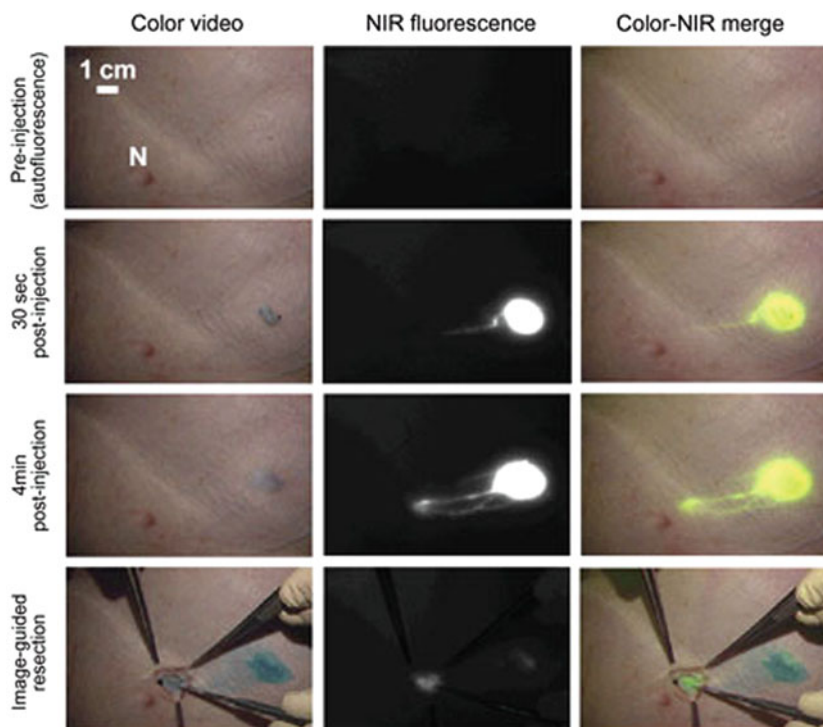


Fig. 9.3 Images of the surgical field in a pig injected intradermally with NIR QDs. From *top to bottom*: before injection (autofluorescence), 30 s after injection, 4 min after injection and during image-guided resection. From *left to right*: colour image, NIR fluorescence and colour–NIR merge images. Reprinted by permission from Macmillan Publishers Ltd: [Nature Biotechnology] 22(1), 93–97, copyright (2003).

Currently, the fluorescent emission and multiplexing capabilities of QDs are being exploited to improve the sensitivity and selectivity in the early detection of tumours. Cancer cells possess surface receptors that can be employed as targets. The first application of targeted NP cancer imaging was carried out in 2002 by Åkerman et al. [123], where peptide-coated QDs with affinity for tumour cells were guided to tumour vasculatures in mice. Later, in 2004, Gao et al. [111] evaluated both conjugated and non-conjugated QDs for tumour contrast in mice, based on active and passive targeting, respectively. Higher sensitivity was observed when QD was conjugated with a specific antibody against a prostate-specific membrane antigen (active targeting). This is in contrast to the emission observed in passive targeting, where non-conjugated QDs are directed to the tumour as a consequence of the enhanced permeability and retention effect. Recently, most of QD/tumour applications are based on active targeting, such as the detection of human liver cancer [124], human glioblastoma tumours [125] or breast tumours in mice [126]. Trends also include the study of the biological processes involved in active targeting, which will allow scientists to improve understanding of tumour biology [20].

Fluorescence optical imaging has been used in living animal models; however, its application is restricted by the poor transmission of visible light through biological tissue. When developing QDs for in vivo applications, a high-fluorescence quantum yield must be maintained after bioconjugation. QDs with NIR excitation (650–900 nm) are thought to be best for optical bioimaging purposes due to the properties of infrared light. Infrared light is of a relatively long wavelength and is therefore scattered less than light of shorter wavelengths. Autoluminescence is also a problem within biological systems. The infrared window between 880 and 660 nm is where Hb, HbO₂ and H₂O absorption is at its lowest [127]. The ability of QDs to withstand photobleaching due to the harsh environments of a biological system and constant irradiation is important and QDs possess an advantage over organic dyes [89]. For a probe to be successful, it needs to be retained long enough to migrate to the required area but also be removed from the system over time [128].

9.3.2.3 Rare-Earth-Doped Particles and Upconverting Nanocrystals

Upconverting nanocrystals (UCNs) are luminophores that absorb light of a wavelength longer than they emit (e.g. NIR light is absorbed and visible light emitted). UCNs consist typically of a LaF₃, YF₃, Y₂O₃, LaPO₄ or NaYF₄ host lattice and are doped with trivalent rare-earth ions, such as Yb³⁺, Er³⁺ and Tm³⁺ [3, 128–130]. The rare-earth lanthanides doped in nanocrystals' centres act as absorbers and emitters of light. For example, the absorber ion (e.g. Yb³⁺) is excited by an infrared light source and then transfers its energy non-radiatively to the emitter (e.g. Er³⁺) ion that undergoes radiative relaxation.

Due to their characteristic excitation and emission properties, UCNs are attractive candidates for many biological applications like drug delivery, DNA (deoxyribonucleic acid) detection, immunochromatography and much more [131–133]. Since luminescent UCNs absorb NIR light, autofluorescence of the sample and/or equipment is minimised. Furthermore, penetration of NIR light into tissue is increased when compared to shorter wavelengths. Figure 9.4 shows an example of in vivo imaging of tissue in small animals using polyethyleneimine (PEI)-coated

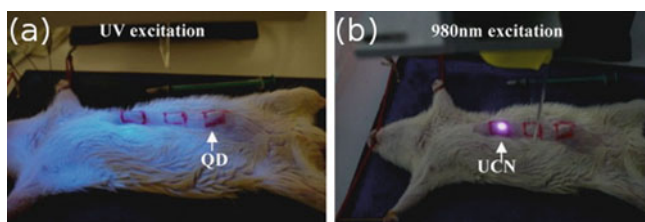


Fig. 9.4 In vivo imaging of a rat with QDs and UCNs injected into abdominal muscle: (a) under UV light source, no emission from QDs can be detected; (b) under IR excitation, the emission of UCNs can be observed. Reprinted from *Biomaterials*, 29/7, Chatterjee, D.K., Rufaihah, A.J. & Zhang, Y, Upconversion fluorescence imaging of cells and small animals using lanthanide doped nanocrystals, 937–943, Copyright (2008), with permission from Elsevier.

NaYF₄:Yb,Er UCNs injected subcutaneously in a rat, displaying high fluorescent detection sensitivity of these particles using continuous wave infrared (IR) laser stimulation [134–136].

Rare-earth and rare-earth-doped NPs represent an exotic class of optical imaging agents [137]. However, the inherent cytotoxicity of heavy elements severely limit in vivo applications [138].

Another emerging class of NPs that have had early in vivo trials are carbon dots [139]. Carbon dots have a potential advantage over certain types of QD and heavy metal-doped NPs as they do not contain any very toxic elements such as gadolinium or cadmium [140]. These particles luminesce through one or two photon excitation processes, although quantum yields are much lower than those of QDs [141].

9.4 Therapy

Nanoparticles (NPs) are in the process of being evaluated as new tools for therapy in biomedical research. This section provides an insight into the three major therapies which employ NPs: hyperthermia, photodynamic therapy and magnetic targeting.

9.4.1 Hyperthermia

Magnetic hyperthermia is an experimental cancer therapy treatment in which target cancerous cells are heated by in vivo NPs beyond their temperature tolerance limits (which are lower than those of normal tissue due to their poor blood supply), thus destroying the cells. This is achieved by exposing the entire patient or the targeted area to an alternating current (AC) magnetic field which will cause the NPs to heat up and ablate the tumour thermally. There are several types of hyperthermia, categorised according to the exposed area or to the applied heating system involved. The most frequently applied techniques are thermoablation and mild-temperature hyperthermia (MTH). Thermoablation kills cancerous cells directly by heating them above 50°C [142], and MTH heats the tumour to 39–42°C for around 1 h, producing immune system stimulation or a heat shock response [143]. More detailed principles and mechanisms of NP hyperthermia are explained in specific reviews (see [144–146]).

The most useful stimuli for non-invasive heating are external activation sources such as microwave, ultrasound applicators, infrared systems and radiofrequency generators used at low frequencies (100 kHz – 1 MHz), which generate an AC magnetic field [144].

In order to implement hyperthermia treatment, magnetic NPs can be introduced in the body through magnetic delivery systems (high.- gradient magnetic fields) or local injection to the affected area [147]. However, permanent magnets (such as NdBFe) are not suitable for in vivo magnetic targeting as just a small portion of magnetic NPs can be held in place [148].

The first in vivo phase II clinical trials of magnetic NP hyperthermia were undertaken in Germany in 2005 [142], by injecting the prostate of cancer patients with biocompatible magnetite NPs. Successful results were obtained using minimally invasive ablation of the tumour in an AC magnetic field after several sessions.

Superparamagnetic particles (primarily iron oxide) of a certain size are favoured for in vivo MTH applications as they have no net magnetisation after an external magnetic field is removed [149]. This eliminates the problem of agglomeration, thus avoiding uptake by the reticular endothelial system (RES) which will remove foreign bodies over a certain size from the bloodstream [54].

Particles of certain sizes are more effective hyperthermia agents than others, so the best NPs are often a compromise between biocompatibility, specific absorption rate (SAR), size and ability to respond to an external stimulus [145]. However, hyperthermia has some limitations as a treatment, which need to be addressed. One of the main drawbacks is that the majority of NPs in ferrofluids do not have high SAR. The required absorption is 10% of the tumour weight in iron, and so thermal ablation of tumours is not always advantageous [73]. In addition, the treatment kills cells indiscriminately at higher temperatures, which is an effect similar to the one observed in chemotherapeutic techniques.

Another emerging technique is plasmonic photothermal therapy (PPTT), which relies upon light to create surface plasmon resonance (SPR) in NPs by photon absorption, thus inducing localised hyperthermia [79]. PPTT has been used in vivo by exposing injected gold nanoshells in the target area to low doses of NIR light, which achieved localised, irreversible thermal ablation of the tumour [73].

Recently, magnetic hyperthermia of NPs has been employed as a key step in novel drug delivery systems by using the heating effects of a magnetic stimulus to rupture the walls of drug-filled polyelectrolyte capsules [150]. Modification of a drug carrier through phase changes brought about by magnetic hyperthermia has also been reported [151].

Exploration into new types of inorganic NPs with different properties compared to the traditional superparamagnetic iron oxide nanoparticles (SPIONs) has been reported. For example, lanthanum manganite NPs with a silver-doped lattice, allows for optimisation of the hyperthermia properties by altering the quantity of silver [152]. Upadhyay et al. [153] reported a holmium-doped magnetite NP lattice, with a very high magnetic moment, with potential use in hyperthermia applications.

Further research must be undertaken to improve several magnetic NP properties. If an optimised NP, in terms of monodispersity, functionalisation, biocompatibility and final size, were synthesised, hyperthermia could be used as a standalone, effective and general treatment of cancer. Currently, NP hyperthermia applications are being implemented in combination treatment with chemotherapy and radiotherapy. In fact, trimodal treatment consisting of NP hyperthermia, cisplatin chemotherapy and radiotherapy has proven effective in phase I and II clinical trials [154].

9.4.2 Photodynamic Therapy

Photodynamic therapy (PDT) is a relatively new method for cancer treatment, which involves the destruction of cancerous cells by the localised production of a reactive oxygen species (ROS), such as singlet oxygen ($^1\text{O}_2$) [155–157]. The ROS is generated by a photosensitiser, which has to be in close proximity to the tumour cells and is usually administered systemically. The major advantages of PDT are that it is relatively inexpensive, non-invasive, can be applied locally and cumulative toxicity effects are not observed. However, the major limitation of this method is the systemic distribution of the photosensitiser and local irradiation of tissue. Advanced disseminated diseases cannot be cured, because irradiation of the whole body with appropriate doses of radiation is impractical. Moreover, irradiation of biological systems is difficult with UV light because of poor tissue penetration and UV-induced tissue damage.

The use of upconverting nanocrystals for the excitation of photosensitisers for photodynamic cancer therapy has several potential advantages. Near-infrared (NIR) light penetrates much deeper into tissue than does ultraviolet or visible light, which allows for the non-invasive application of the method. Furthermore, NP surfaces can be functionalised – e.g. targeting moieties can be coupled covalently as described in Section 9.2. In principle it is possible to construct a non-invasive, highly specific drug for photodynamic cancer therapy that enables “automatic” concentration at tumour sites. This allows for the treatment of any tumour or metastasis which can be illuminated with NIR light, despite systemic administration of the drug.

Zhang et al. [158] reported large, coated (~ 100 nm diameter), upconverting $\text{NaYF}_4:\text{Yb},\text{Er}$ NPs with a porous silica layer, with embedded with merocyanine 540 (photosensitiser) molecules. The generation of singlet oxygen was observed in these findings, but in vivo experiments were not pursued. One year later, Chatterjee and Yong [159] published a similar paper, where the photosensitiser zinc phthalocyanine was used. It was shown that the NPs were taken up by cells and irradiation with NIR caused cell death. Recently, Qian et al. [160] published very similar results to Chatterjee et al.

In conclusion, it has been shown that photosensitisers can be excited with upconverting NPs (UCNs) and that these drugs are capable of producing $^1\text{O}_2$ and other ROS. However, the published systems have not been utilised for photodynamic cancer treatment. There are two major drawbacks concerning the UCNs: their large size and the thickness of the silica shell. The NP size was between 50 and 120 nm, which is too large for biomedical applications. The $^1\text{O}_2$ /ROS was produced within a relatively thick porous silica layer on the surface of the upconverting NPs and likely to be degraded prior to diffusion out of this shell. Despite these drawbacks, and considering that this method is still in its infancy, it has tremendous potential for cancer therapy.

9.4.3 Magnetic Targeting

A major drawback of cancer chemotherapy is the non-specific delivery of cytotoxic pharmaceutical agents. Drug delivery via the entire body commonly leads to undesirable side effects. For example, patients suffering from arthritis may be forced to curtail their treatment because of acute inflammation in healthy joints.¹⁶¹ The use of magnetic NPs to target specific areas is an emerging solution to avoid side effects. In principle this could enable the application of multiple drugs which currently cannot be administered together. Specific drug targeting involves loading a cytotoxic drug onto SPIONs that are then injected intravenously and directed to the target area by applying a magnetic field gradient. Finally, the drug is released in the selected area using a chemical stimulus such as altering the pH, temperature or by the use of enzymatic reactions [54, 161, 162]. This method allows for a significant reduction of the overall drug dose due to it being localised at the site of treatment. In turn, this reduces systemic side effects.

There are two main types of magnetic carriers: single SPIONs bioconjugated with biocompatible polymers such as dextran or starch [162–165] and porous biocompatible matrices of polymer or silica embedded with SPIONs [161, 162, 166, 167]. Recently, more exotic and original magnetic drug delivery systems have been reported [168, 169].

Drug loading and release can be achieved in a variety of ways. A simple scheme involves the electrostatic complexation of the drug onto the oppositely charged surface of coated SPIONs [163]. The drug is then released by desorption upon pH variation or simply upon dilution [164]. More robust strategies are based on the use of stimuli-responsive polymers. For example, release of doxorubicin encapsulated within thermo-responsive polymer containers can be triggered by a temperature or a pH stimulus [168]. pH-switchable nanocarriers are particularly useful, especially in endocytosis, as cancer cells have a slightly lower pH than healthy ones [168]. Drugs can also be embedded into biodegradable polymers or covalently coupled to the surface of the coated SPIONs through peptide bonding [166, 170, 171]. The drug is subsequently released by intracellular enzymatic reactions. Recently, Hu et al. [169] reported the synthesis of a complex core–shell nanostructure for drug delivery under a high-frequency magnetic field (HFMF) (see Section 9.4.1) stimulus, with simultaneous in situ monitoring. The core consisted of a polymer matrix in which the drug molecules were embedded, with the external shell consisting of a thin layer of single crystals of iron oxide. Additionally, QDs were attached to the magnetic shell for optical imaging purposes. Upon application of a HFMF, the magnetic shell was subjected to lattice distortion which led to the formation of nanochannels, which allowed for partial drug release. As the width of the channels increased, shell integrity was lost, leading to full drug release.

Even though the most advanced magnetic drug carriers are still in developmental and in vitro testing stages, in vivo experiments with simple drug-loaded SPIONs have proven the viability of magnetic targeting principle. To date, several studies have reported successful cytotoxic drug delivery and cancer remission in

rats, rabbits and swine [164, 172–177]. For example, Lübke et al. [172] implanted malignant and highly metastasising carcinomas into the ears and abdomen of mice. Starch-coated SPIONs functionalised with the cytotoxic drug epirubicin were injected into the tail vein, while an external magnetic field was applied in the tumour vicinity. Cancer remission was achieved with the injection of a SPION ferrofluid equal to 0.5% of blood volume. Side effects were minimised, although the SPION concentration reached 10–20% of blood volume. In 1996, the same authors performed a clinical trial on a group of 14 patients with various types, localisation and stages of solid tumours [178]. After injection of the cytotoxic drug-loaded SPIONs and application of a magnetic field for 60–160 min, six patients presented an accumulation of SPIONs within the tumour tissue. Unfunctionalised SPIONs were well tolerated, whilst drug-loaded ones induced minimal side effects. This method was assessed to be safe for use in cancer therapy, even though embolisation has been reported as a risk in several studies.

Despite this success, the transfer of magnetic drug targeting from animals to humans presents difficulties. The magnetic attraction of SPIONs to targeted area depends linearly on the applied field, the gradient of the field, the volume of the magnetic particles and their magnetic susceptibility [161]. The limited availability of external magnets reduces the field penetration depth to 10–15 cm [54, 161, 162]. Another limitation arises from the fact that higher blood flow rates require either higher strength fields or higher field gradients for magnetic targeting to be effective [161]. So, magnetic targeting can be used only in regions with slow blood flow rates.

The aforementioned limitations could be overcome by the use of magnetic implants, which were developed initially to target complications occurring near surgical implants. The implants combine with soft ferromagnetic materials that attract healing SPIONs injected into a neighbouring artery [54]. In vivo experiments were conducted on dogs, which were implanted with prosthetic carotid which acted as a magnet, producing encouraging results [179].

Magnetic targeting is being developed in parallel with biochemical targeting which uses specific recognitions to target cancer cells. Recently, Kim et al. combined magnetic and folate-targeted doxorubicin delivery to cancer cells [166, 180]. The use of magnetic and folate targeting increased the cytotoxicity when compared to folate targeting alone. A combination of both methods will certainly be exploited in future applications.

9.5 Toxicity

This chapter aims to provide the reader insights into the wide range of potential diagnostic and medicinal applications achievable using inorganic nanoparticles (NPs). As for any diagnostic or medicinal tool, it is only prudent to assess their toxicity for further in vivo applications on humans. While these investigations are only at an

early stage, it appears that alarming conclusions are already being drawn, especially by the public media. Additionally, recent reviews on the toxicity of nano-engineered materials point consistently at contradictory conclusions between toxicological reports and call for more comparable experimental data. These discrepancies stem from the tremendous complexity of the investigated systems. In this section, the toxicity will be approached from a number of perspectives to help construct a reliable toxicity assessment. We will focus on conditions that are relevant to the *in vivo* applications described earlier in this chapter. Hence, toxicity issues related to the inhalation of nanomaterials or exposure to nanomaterials during their synthesis will not be reviewed here (else see, e.g., [Chapter 18](#)).

9.5.1 A Complex Task

With 66 reviews published in 2009 compared to 5 in 2005, the assessment of the NP toxicity is attracting a growing interest. Amongst the accounts it is noticeable that results of recorded cellular damages for given families of NP appear to be inconsistent. To illustrate this, cadmium selenide [[181](#), [110](#), [182](#)], iron oxide [[183–188](#)], gold [[75](#), [187–190](#)] and silica [[188](#), [191–193](#)] NPs have all been reported to be either toxic or non-toxic. These polarised conclusions originate from the fact that there is currently no standard protocol for the assessment of the toxicity of nanomaterials. Among these studies, the experimental parameters vary to such an extent that in most of the cases, reliable comparisons are known to be improbable. The goal of Section [9.5.1](#) is to draw guidelines for the critical examination of the toxicity assessment of nanomaterials.

9.5.1.1 Practical Considerations

Precise toxicity assessments emerge from the concepts of cytotoxicity or genotoxicity. Inorganic NPs have been reported to be cytotoxic through the triggering of inflammatory responses [[187](#), [194](#)], oxidative stress [[187](#), [188](#), [190](#), [195–198](#)] or ion signalling disruption [[199](#)].

If their size in the cell medium is small enough, inorganic NPs are able to enter the nucleus. Thus, genotoxicity can occur through damaging of deoxyribonucleic acid (DNA) by inflammation [[188](#)], oxidative stress [[186–188](#), [198](#), [200](#)] or by direct interaction with DNA [[188](#), [190](#), [198](#), [200](#), [201](#)]. NPs have also been proven to be genotoxic through epigenetic mechanisms. Such mechanisms include altering the transcription/expression of DNA repair genes [[188](#), [190](#)], cell-cycle regulator genes [[187](#), [202](#)] or mechanically inhibiting proteins responsible for replication, transcription and cell proliferation [[203–205](#)].

9.5.1.2 Experimental Set-up

The geno- or cytotoxicity of nanomaterials is found to be strongly dependent on the cell line tested. Tkachenko et al. [[206](#)] compared the four peptide–BSA–gold

conjugates in three cell lines: HeLa, 3T3/NIH and HepG2. Each cell line exhibited different cellular and nuclear uptake capacities. Variation of uptake is due to the dose size and the mechanisms responsible for toxicity. Hence the cell line should be taken into account when drawing conclusions about toxicity as uptake differs between cell lines.

The toxicity of a given nanomaterial is influenced by the following parameters: culture conditions for in vitro studies, the method of administration for in vivo studies, NP concentration and time exposure. Unfortunately, the range over which these parameters have been observed has not been standardised. Time exposure and NP concentration have been used over such a wide range in the literature that it is difficult to determine whether the observed toxicities are physiologically relevant [187].

In the literature, there are several discrepancies between the results of toxicity assays performed with different testing methods on the same nanomaterial [188]. In addition, there are sources of discrepancy that stem from the nature of the nanomaterial under scrutiny.

In most of the studies, cell death is investigated using colourimetric assays, either based on absorption or emission. The neutral red and trypan blue tests provide evidence of the signs of cell death, which is leakage into the cell membrane. However, most inorganic NPs that are useful for in vivo applications are themselves strong light absorbers or emitters. These optical properties may affect the detection of the testing dyes adversely through optical phenomena such as fluorescence resonance energy transfer (FRET) [207, 208]. Therefore, this may lead to disparity of toxicity results [198].

Another important issue is that an interaction between the test dye and the surface of the NP can occur. If this was the case, then the interaction between NP and dye may occur in parallel or instead of the interaction of the dye and the target biological material. This would result in a false-positive result [187, 209].

Common cytotoxicity or genotoxicity assays exist to probe the effect of chemical compounds that can readily diffuse into cell over period of time relative to their half life. As a consequence, genotoxicity assays are rarely run for more than 24 h. However, the low mobility of inorganic NPs as compared to that of molecular compounds may lead to extended uptake and cellular movement times. Hence, several studies have expressed the need to extend the time exposure of the common tests when dealing with nanomaterials [188, 210].

The chemical structure of the NPs is thought to contribute to the discrepancies in published results. There is a general agreement that the NP shape, size and coating have a large influence on the toxicity [187, 188, 198, 211, 212]. Numerous toxicological reports are based on ill-defined nanomaterials. The latest reviews call unanimously for consistent and comprehensive physico-chemical characterisation of the NPs to be investigated. These features of the toxicological literature contrast sharply with the latest achievements of complex nano-architecture or multi-functional nano-platforms [213–217]. Hence, there is a desperate need for a share of expertise from the biologists and material scientists.

9.5.2 Towards Measuring Toxicity: Chemical and Nanoscopic Risks

Bearing in mind the aforementioned guidelines, we now propose a classification of the causes of toxicity resulting from inorganic NPs into chemical and nanoscopic risks.

9.5.2.1 A Chemical Risk

Prejudice regarding the toxicity of nanomaterials is often related to their size, which potentially allows for cellular or nuclear uptake. However, it appears from recent reviews that the main cause of their toxicity originates from a chemical source; the inorganic core or the surface coating.

One important source of toxicity is the cell exposure to intrinsically cyto- or genotoxic metal ions lost from the NPs or direct contact with their surface. Cytotoxicity of cadmium-containing quantum dots (QDs) has been found consistent with cytotoxicity of free cadmium, a known oxidative stress inducer, released from the NP, e.g. through oxidation of the NP surface [198, 218]. Cytotoxicity of iron oxide NPs has been found to arise from Fenton or Haber Weiss oxidation reactions induced by the iron-rich core [188, 196, 197]. NPs are routinely coated with inorganic shells, ligands or polymers to impart solubility in biological media. The NP coating can isolate the reactive metal ions of the core from the cell and can thus prevent oxidative stress. Encapsulation of CdTe or CdSe QDs with a zinc sulphide shell decreases their toxicity dramatically [198, 218–220]. Coating of iron oxide NPs with a polysaccharide suppressed the cytotoxic effects observed with the bare NPs [184, 187, 221].

Besides providing NPs with water solubility and targeting functionality, bioconjugate coatings can be responsible of their cyto or genotoxicity. Several reports have attributed the cytotoxicity of bioconjugated NPs to the cytotoxicity of the coating ligands themselves [20, 187, 198, 222, 223] (Fig. 9.5).

As exposure of the metal ions or release of free ligands can trigger cell damage, the stability of the bioconjugated NPs is essential to prevent cyto or genotoxicity. The resilience of the coating layer to oxidative stress over long period of time is of particular importance, especially if bioaccumulation or slow evacuation is to occur. In the case of cadmium-based QDs, the zinc sulphide shell has been found to deteriorate over time [198, 202, 224]. Moreover, shelled QDs can still generate free radicals that could eventually digest the bioconjugate layer [188, 220]. This would ultimately lead to the release of free cadmium and thus to oxidative stress.

9.5.2.2 A Nanoscopic Risk

This section focuses on so-called nanotoxicity which is defined as “the ability of a substance to be cytotoxic owing to its size and independent of its constituent materials” [89]. We aim at showing that the size acts mainly as a regulator of the

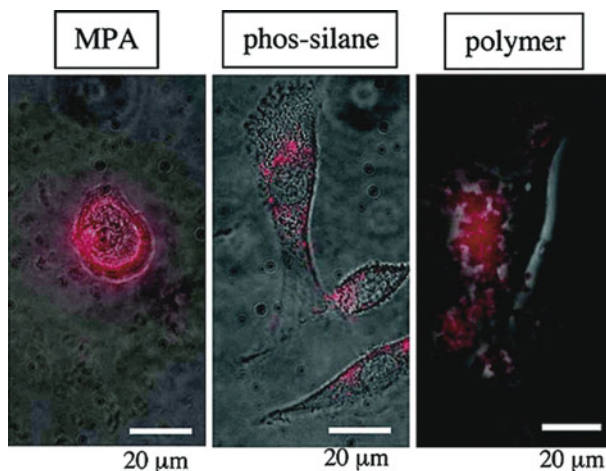


Fig. 9.5 Comparison of different surface particle incubated for 48 h with high concentrations of CdSe/ZnS QDs: for mercaptopropionic-coated (MPA) QDs, cells are dead, while the cell debris remains on the substrate. For silane-coated QDs, no effect of the particles on the cells can be observed; the particles are ingested and stored around the nucleus and cells remain adherent. Polymer-coated QDs tend to precipitate on the cell surface, and most cells detach from the surface, while the few still adherent cells are alive. Reprinted with permission from Nano Letters 2005, 5(2), 331–338, Copyright 2005 American Chemical Society.

above-mentioned chemical risks, though some toxic mechanical effects specific to nano-sized objects have been shown.

In general, the smaller the NP, the greater the toxicity [187, 225]. This is due in part to the fact that small NPs are more readily uptaken into the cell or even the nucleus. Larger NPs may therefore be less cytotoxic simply because their cellular uptake is limited [206]. If uptake occurs, the varying size of NP will result in a change of magnitude of cytotoxicity because of the difference in the chance of triggering cell death. For example, Lovric et al. observed that red CdTe QDs of diameter 5.2 nm accumulated in the cytoplasm, whilst green QDs of diameter 2.2 nm were found predominantly in the nucleus [187, 226]. The QDs of diameter 2.2 nm were found to be more toxic, what has been attributed to the possibility of damaging DNA.

As the NP size decreases, the surface-to-volume ratio increases. Hence for cytotoxicity involving reactions at the surface of the NPs, such as oxidation of cadmium selenide, the smaller particles will have a greater toxic effect. This combined with the increased uptake of smaller NPs will result in higher doses of free cadmium ions. Therefore, it has been suggested to replace the NP mass by the specific area as a standard dose parameter in cytotoxicity studies [212].

In most cases, toxicity increases as the concentration of nanomaterial injected or introduced in the culture medium increases. This is in keeping with the increase in dose. However, Auffan et al. have reported an increase in cell tolerance upon exposure to higher NP concentration [186, 188]. This finding was attributed to a

doubling of the effective size of the NPs through aggregation at high concentration, which eventually resulted in weaker uptake.

The nature of NP size is expected to have significant influence on cytotoxicity through their association with cellular macromolecules or organelles. Investigations of these interactions are in their infancy [227]. It has already been demonstrated that NPs can form complexes with proteins, such as histone, and may consequently alter their functions [204]. It is also to be expected that NP aggregates sterically hinder or alter the functions of organelles like cytoskeleton and extrusion vesicles.

9.5.3 Conclusion

The toxicity of nanomaterials is at the heart of a vast research effort. It is often very difficult to draw conclusions by comparing early toxicity studies, although they have still been informative. They have allowed for the identification of important parameters which trigger toxicity.

There is now a unanimous call for standardisation of assessment protocols for toxicity. This standardisation is not out of reach on a practical point of view and if it were to occur, it would greatly accelerate understanding and development of toxicological trends with nanomaterials.

When toxicity is observed, it has been found to arise from parameters which are not currently controllable, such as NP stability or dose. Therefore, it is unlikely that toxicity issues should hinder the development of the exciting bioapplications presented in this chapter.

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Chapter 10

Cell Cultivation and Sensor-Based Assays for Dynamic Measurements of Cell Vitality

Angela M. Otto

Abstract Cell cultivation is a fundamental tool in tissue engineering as well as in biomedical research. Choice of cell source and the control of cultivation parameters will determine the biological relevance and quality of the results. There are numerous biochemical and cellular assays available to test the vitality, i.e. the metabolic and functional activities, of cells in culture. Most of these assays, however, are end-point measurements and give information only for a selected time point. For non-invasive real-time measurements on cells or tissue cultures, multiparametric sensor chip test systems have been developed. They have in common: (1) sensor arrays for monitoring changes in extracellular acidification and O₂ consumption, and optionally, electrodes for impedance; (2) integration of the sensor chip into cell culture containments; (3) a fluidic system to provide cells with fresh medium at regular intervals, which is a prerequisite for detecting metabolic changes and allows the addition and removal of test solutions; and (4) continuous signal monitoring in a non-invasive manner for prolonged times. The sensors are either electric (e.g. ISFETS, metal oxides, Clark-like electrodes) or opto-chemical (fluorescent dyes), the latter being used in 24-well systems. These test systems are being applied for analysing the metabolic activity in various cell types, including pancreatic islets and β -cells, with regard to their energy metabolism and insulin secretion. The data could also serve top-down approaches in systems biology in providing functional information.

Keywords β -Cells · Cell culture · Energy metabolism · Extracellular acidification · Multiparametric sensors · Insulin secretion · Metabolic assays · Oxygen consumption

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Abbreviations

ATP	adenosine triphosphate;
ELISA	enzyme-linked immunosorbent assay
FADH ₂	flavin adenine dinucleotide-reduced
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid
IDES	interdigital electrode structures
ISFET	ion-sensitive field effect transistor
LAPS	light-addressable potentiometric sensors
NADH	nicotinamide adenine dinucleotide-reduced

10.1 Introduction

10.1.1 Cell Cultivation in Biomedicine

The cultivation of cells is an intricate procedure for tissue regeneration as well as basic research in biomedicine. In scientific projects, the cell culture serves several functions: it provides experimental material which is easier to handle than animals; it reduces the levels of complexity by being restricted to one (or only a few) cell type(s); it generates cell populations with less biological variability than a complete organism and thus gives a much better reproducibility of results; and it allows the application of a greater variety of investigative tools. However, the loss of the native physiological environment of the cells results in the loss of contact to other cell types, the extracellular matrix and a specific mixture of growth regulatory factors and hormones, and can have dramatic effects on cellular morphology and functions. Examples of such transformation are changes observed as alterations in the expression of cytoskeletal components as well as in regulatory mechanisms of cell-specific functions, often resulting in cellular regression (dedifferentiation). These drawbacks should be kept in mind when using cultured cells.

The objective of tissue engineering as a constituent of regenerative medicine is to provide replenishment or replacement of diseased or wounded tissue. This requires the recruitment of appropriate cells as well as suitable culture conditions. Precursors for such cultures can be primary cells, i.e. cells directly isolated from either auto- or allogenic tissue, or stem cells from the tissue of the patient [1]. Also, these cells may grow on matrices or scaffolds for implantation. The most demanding task in cultivating cells is to maintain their growth characteristics, namely their metabolic activity and specific functions. The standards for the quality of cell cultures and the expected information obtained with a myriad of methods are accordingly high.

To select for the appropriate analytical parameters, it is important to define some ambiguously used terms: *Growth* means the increase in size of a tissue, organ, or organism. This can be achieved by an increase in cell number, but also by an increase in the size of individual cells, e.g. of fat cells. At the cellular level, growth usually

refers to a cell number, but may also mean cell mass, usually cellular protein content. An *increase* in cell number will be the result of cell division (cell proliferation). A *decrease* in cell number needs to be explicitly defined: It can mean that the number of cells has decreased over the initial number, usually as a result of cell death. But, it can also be that the increase in cell number is lower than expected, e.g. compared to a control culture. Such a growth inhibition may be the result of an attenuated rate of cell proliferation, or of concomitant proliferation and cell death in the cell population. A commonly used term to describe cell growth or proliferation is *viability*. As the origin of the word implies, it means the capability to live, grow, and function. This term is usually used when testing for cytotoxicity or other growth inhibitory effects. However, in the contexts of tissue engineering, the key issue is: how *active* are the cells, what do they make of their capabilities? Here, the suitable word to describe the state of cells is *vitality*, which means the capacity to perform life-sustaining functions, or the state of metabolic and functional activity.

Cellular *function* is the activity that fulfils the specific purpose of a cell. This can be cytoskeleton contraction for mechanical work (muscles), transmission of information (nerves), nutrient transport (gastric epithelial cells), or secretion of insulin (β -cells), to name just a few examples. Therefore, the test parameters for function will depend on the processes involved in the specific cell type. On the other hand, parameters for vitality will be common to many cell types.

It is the goal of this chapter to provide some biochemical background to help understand the basics of frequently used biochemical and cellular test procedures and, in particular, metabolic sensor chip-based assays. Moreover, some basics of cell cultivation with respect to its advantages and limitations will be introduced, since this is the groundwork for obtaining functional data which could be amenable to systems biology.

10.1.2 Biochemical Processes Describing Cell Vitality

All animal cells are endowed with remarkably conservative metabolic pathways for producing and transforming energy; and energy is obviously a prerequisite for performing any kind of cell function. A common currency for dealing with energy is ATP, a nucleotide with energy-rich bonds and a partner in uncountable biochemical reactions in the cell. Some key reactions involved in deriving energy from metabolites, beginning e.g. with glucose, are shown in Fig. 10.1. The metabolism of glucose to pyruvate, i.e. glycolysis, will alone produce two ATPs per glucose. At this point, two types of reactions can proceed: Under *aerobic* conditions, i.e. in the presence of normal oxygen concentrations, pyruvate is shuttled into the mitochondria, where it will be further metabolized in the tricarboxylic acid cycle (Krebs cycle), ultimately producing CO_2 and yielding hydrogens transferred via NADH or FADH_2 , two components of the respiratory chain. While H^+ goes into the intramembrane compartment of the mitochondria, the electron remains with the proteins of

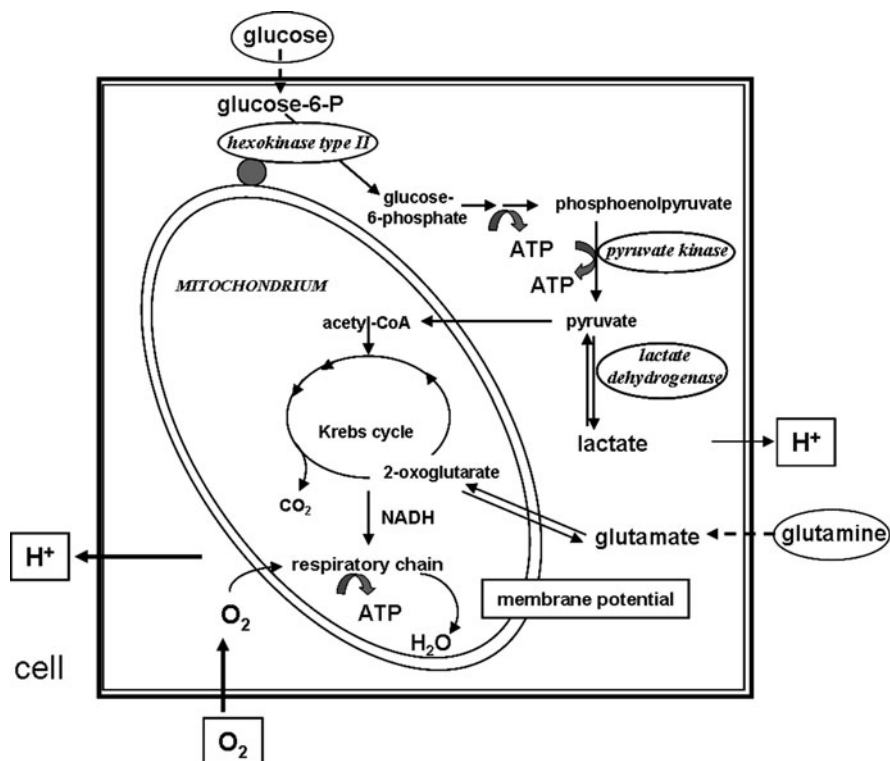


Fig. 10.1 Scheme of key reactions of energy metabolism in animal cells relevant for cell vitality

the respiratory chain. This dissociation of proton and electron from hydrogen leads to an increase in mitochondrial membrane potential and is a driving force for the subsequent reaction of oxygen and hydrogen to water in an enzymatically controlled way via the ATP synthase – where the energy is conserved in form of ATP (see biochemistry text books; essentials are compiled by Owicki and Parce [2]).

Under *anaerobic* conditions, pyruvate mainly converts to lactate by a single reaction catalysed by lactate dehydrogenase. Lactic acid is expelled from the cell via monocarbonic acid transporters. This reaction along with the transport of other acids originating from the Krebs cycle is responsible for the acidification of the cellular microenvironment.

Another key metabolite in energy metabolism is glutamine. It is deaminated (releases its ammonium groups) to glutamate and then oxoglutarate, which is a component of the Krebs cycle. Through this pathway, glutamine can be converted to pyruvate. Since these reactions occur in the mitochondrion, they directly fuel the respiratory chain.

A number of components of these pathways related to the energetic state of the cell are suitable for assaying cell vitality, e.g.

- the level of ATP
- the activity of mitochondrial dehydrogenases
- changes in mitochondrial transmembrane potential
- the rate of acid extrusion
- the rate of O₂ consumption

These can be considered as general output parameters of cellular response to numerous biochemical stimuli, toxic agents, and changes in the culture environment. In particular, the *rate* of extracellular acidification and oxygen consumption are good candidates for electrochemical and optical sensors, which have been developed and refined for these purposes and will be described in Section 10.4.

10.2 Prerequisites for Assessing Cell Vitality and Function In Vitro

10.2.1 Cell Culture Conditions

The most demanding task in maintaining the characteristic features of a cell or tissue in vitro is providing the proper cultivation environment. This is essential to ensure not only reproducible, but also biologically meaningful data on cellular processes. Moreover, such conditions should be standardized, so that experiments can be repeated and compared with those in other laboratories. The basics of cell cultivation are described in classical method books, which are a good introduction and guide line [3, 4]. The list of essential requirements gives an idea of the uncountable variations possible (Table 10.1).

10.2.2 Culture Conditions for Islets and β -Cells

While islets isolated from the pancreas are three-dimensional cell compounds, β -cells, either as primary cells isolated from islets or established as cell lines, usually grow as two-dimensional clusters in normal tissue culture flasks. To enable a more tissue-like three-dimensional arrangement, β -cells are also cultured as spheroids (pseudoislets) in suspension. Due to the restricted availability of human islets for research and the lack of human cell lines, most laboratories work with islets or cell lines obtained from different animal species. Common cell lines are, e.g., HIT-T15 (hamster), MIN6 (rat), β TC-tet (mouse), or INS-1 (rat). Of these the INS-1, derived from a rat insulinoma, and its cloned sublines such as the INS-1E, which was selected for its insulin secretion response, are considered to be the most representative ones for different states of insulin production in resembling that of isolated (rat) islets [5, 6]. This makes these latter cell lines a good model for studying β -cell function at the biochemical and cellular levels.

Table 10.1 Some essentials to be observed in cell cultivation

Cells	Origin (species, tissue; history) Source received from (commercial, other laboratories) State of differentiation
Basic culture medium	Components (amino acids, salts, vitamins, co-factors, metabolites) pH Concentrations of components Osmolarity
Supplements	Serum (species, age, pre-treatment) Serum surrogates, biological extracts Growth factors (including insulin, hormones, cytokines) Buffers (NaHCO ₃ , HEPES ^a) Antibiotics
Growth surface	Tissue culture plastics, glass Extracellular matrix components Synthetic polymers (e.g. poly-L-lysine)
Propagation protocols	Cell concentration, density Medium changes Time between transfer to new culture vessels (depending on growth rate of cells)
Incubation environment	Temperature pCO ₂ pO ₂

^aHEPES, a non-volatile synthetic organic buffer with a pK_a of 7.5.

In most recent publications, isolated islets as well as the β -cell lines are cultivated in the same basic medium, RPMI 1640, containing 11.1 mM glucose and 2 mM glutamine. This medium was shown to be superior to others tested with respect to the stimulation of insulin production of islets in cell culture [7]. For cell lines, 5–10% fetal calf serum (FCS) is used to maintain cell adhesion and prolonged growth. The medium for INS-1 β -cell lines is also supplemented with 50 mM 2-mercaptoethanol, 1 mM pyruvate, and 10 mM HEPES. For the cultivation of islets there are some variations in medium and supplementation, including the use of serum-free medium. A low glucose concentration (3–6 mM) has been shown to reduce energy metabolism of islets and doubling time of INS-1E cells, respectively [8, 9] as well as static insulin release [10]. The concentrations of glutamine, as well as leucine, ranging from 0.02 to 20 mM are also variables found to influence the energy metabolism of β -cells, especially in connection with prolonged cultivation at low glucose concentrations [11, 12]. Therefore, changes in medium composition alter not only the basic metabolism of the cells, but will also affect their secretory activity (see [Chapter 3](#)).

To improve the viability and functional activity of isolated islets in culture, different extracellular matrices and growth substrates are being investigated. Islets cultivated on components such as collagen, laminin, and hyaluronic acid show better survival and higher activity of insulin release [13, 14].

The bottom line is that if data from different studies are to be comparable and amenable to systems biology, cell culture and stimulation conditions to be used for vitality and functional testing need to be prudently selected, explicitly documented, and, where possible, standardized.

10.3 Biochemical Assays and Their Information

10.3.1 Testing Cell Vitality

To characterize and quantify the energetic state of cells in culture, an arsenal of methods exists, many of them commercially available as kits, i.e. with instructions and all components provided in a standardized form. A selection of assays is listed in Table 10.2. As discussed above, the choice of the method will depend on the type of information required and the technical facilities available.

Table 10.2 Some common assays for testing the viability and vitality of cell cultures

Assay principle	Method	Apparatus	Limitations
Cell proliferation	Cell, nuclei counting	Microscope	Small numbers
	Impedance	Electronic counter	Distinction of dead cells
	Cell cycle markers	Flow cytometer	Laborious, indirect
Cell mass	Protein content (1)	Photometer	Interference by biochemicals
	DNA content	Fluorescence spectrometer	Toxic labels for detection
Viability	Live/dead fluorescent assay	Flow cytometer	Laborious and time-consuming
	Trypan Blue exclusion	Microscope or photometer	Small numbers
Mitochondrial activity	Tetrazolium-based assays (2)	Photometer (ELISA reader ^a)	Biochemical basis ill-defined
	AlamarBlue (3)		Cell toxicity
Bioenergetic status	ATP-luminescence (4) NADH	Luminescence reader Photometer	Chemical solubilization of cells required

^aELISA reader is a commonly used name for a photometer which was originally developed to measure absorbance in a 96-well plate used for enzyme-linked immunosorbent assays (ELISA). Most standard methods for cell analysis are described in cell culture manuals. Many biochemical-based assays are available as commercial kits; some commonly used kits are referenced to exemplify also possible limitations and the specific information they can provide. References: (1) [39]; (2) [40]; (3) [41]; (4) [42].

10.3.2 Testing Cell Functions

The method of choice for measuring cell function will obviously depend on what the cell is expected to do or produce. Some functions, as e.g. stimulation of insulin

secretion, can be dissected into several steps, with each step requiring a different method of analysis [15]. Also, many functions are intimately connected to energy metabolism, meaning that the dynamics will depend on the available metabolite or ATP levels [16]. In the context of hormone-producing cells, the methods can range from the detection of electrical changes at the level of the cell membrane, e.g. by patch clamp, to quantifying the end product released, e.g. a cytokine or hormone by immunological assays.

10.3.3 Stimulating Insulin Secretion in Islets and β -Cells

A common protocol for stimulating insulin secretion in culture [5, 8] is to preincubate cells for about 2 h in culture medium or Krebs-Ringer bicarbonate HEPES (KRBH), a buffer without glucose to downregulate the signals for insulin secretion and make the cells sensitive to high glucose exposure. After this depletion of glucose, the cells are returned to a solution with high, usually 11.1–16.8 mM, glucose, which in healthy β -cells stimulates a rapid release of insulin. The maximum of insulin release in islets is observed after 5–10 min; thereafter insulin secretion declines, but may persist at lower levels for several hours [15]. Generally, glucose-stimulated insulin secretion (GSIS) is measured after 30 min. The two presently available methods of determining insulin secretion are based on appropriate antibodies, which specifically bind insulin and are quantified by a radioimmune assay (RIA) or an enzyme-linked immunosorbent assay (ELISA).

There are several variables in the stimulation protocol. Obviously, the levels of insulin secreted will depend first of all on the level of glucose to which the cells were exposed prior to stimulation. A high glucose level transiently leads to an enhanced rate of basal insulin release [10]. Also, extracellular glutamine as well as intracellular glutamate (both up to 20 mM) can enhance the secretory activity of β -cells [12, 17]. A critical point is the fact that the complete removal of glucose and amino acids prior stimulation will rapidly alter energy metabolism [18]. But also serum deprivation has numerous effects, including a rapid increase in the degradation of long-lived proteins [19] and the induction of apoptosis [20]. Altogether, these parameters will affect the biochemical processes regulating insulin production, storage, and release, and should be well considered in the protocol.

10.3.4 Defining the Time of Measurements

Many of these biochemical assays allow testing a probe only once (methods of “no return”), since either the cells are sacrificed to obtain access to cellular components, e.g. for antibody-labelling, or they need to be treated with eventually toxic agents. Even live-cell fluorescent labelling, while being a dynamic measurement albeit for a short time in the range of minutes up to a few hours, will ultimately disrupt cellular processes. These methods do not allow following the dynamic development of a cellular process in an individual sample for a prolonged time. Therefore, as

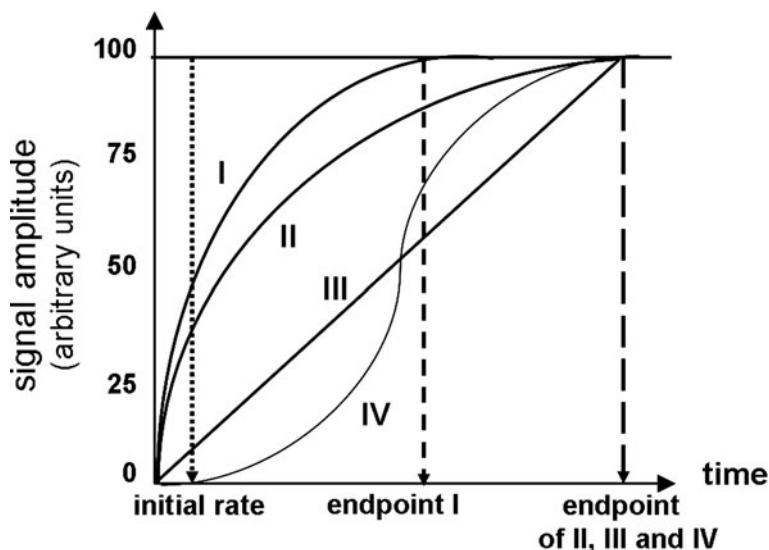


Fig. 10.2 Choosing time points for measurements requires knowledge of the underlying kinetics of the activity or reaction assayed. The four hypothetical curves here show different kinetics for measurements of cellular activities and different time points at which the maximum activity is reached. An early time point for measurements representing the initial rate of a process will require knowledge of its kinetics. Note also that the curves I and II have different end points, while curves II, III, and IV may have the same end point in spite of having different kinetics. Curve IV could represent a growth curve or, at the molecular level, a cooperative process.

illustrated in Fig. 10.2, the timing of an experiment will determine at which stage a cellular process is analysed; and the time of measurement is obviously paramount for interpreting biological data. To obtain information on the kinetics of metabolic or functional changes at the level of live cells, either many probes are needed to measure a dynamic process at various times or methodologies for non-invasive dynamic measurements on the same cell sample are required.

10.4 Dynamic Measurements Via Multiparametric Sensor-Based Assays

10.4.1 Basic Properties

As alluded to in the previous sections, energy metabolism of animal cells has common enzymatic and regulatory components, thereby making these suitable candidates for monitoring the dynamics of cell vitality. For metabolites involved in energy metabolism, different types of sensors for dynamic measurements have been developed, e.g. for glucose, lactate, CO_2 , O_2 , and pH. Two processes best accessible for sensor detection are the acidification of the medium and changes in oxygen

concentration. The advantage of being able to monitor two or more parameters simultaneously is that one can obtain multiple sets of information from the same cell population on processes which may be under different controls and, therefore, may behave differently to external conditions. For this reason multiparametric sensor chips with two or more different sensors have been developed [21].

Such sensor-based assays for dynamic measurements of the metabolic and functional activities of cells and tissues have the following features in common:

- *One or more sensors*: These are of a biocompatible material, are non-invasive, and can be combined for parallel detection on the same probe.
- *Cell culture integration* of the sensors: the sensors are part of the cell culture, either as an integral part of the growth surface of a well or immersed into the culture medium.
- *Life support*: A fluidic system allows maintaining the cells under cell growth conditions for up to several days or longer. In general, the standard culture medium will include serum, but it needs to be without added buffer (NaHCO_3 , HEPES, etc.) to ensure the sensitive detection of pH changes in the medium. A complete exchange of the cell conditioned medium at regular intervals ensures the ample supply with required nutrients and oxygen, while removing extruded metabolites and acids which could attenuate cell vitality. Furthermore, a regular exchange of medium in the culture is the prerequisite for being able to measure the *rates* of acidification and oxygen consumption – rather than an accumulative effect. Some test platforms also have the option of directly adding and/or removing test agents (e.g. stimulants or drugs) with the medium.
- *Continuous signal monitoring*: Since the sensors are non-invasive, i.e. do not interfere with the functional activity of the cells or tissues, the measurements are in real time.

10.4.2 Electric Sensors

In the biochemical laboratory, pH and O_2 sensors have long been in use, albeit at a macroscopic level for test tubes and beakers. The challenge of developing electrodes for measuring pH and O_2 in cell culture was their miniaturization, biocompatibility, and stability as well as the integration of different sensor types onto a common platform. Of the different developments, two main classes of electric microsensors have led to *multiparametric arrays* for in vitro cell measurements: sensors based on silicon technology and sensors based on thin film technology. These will be briefly described below.

In *silicon technology* two types of pH sensors have been developed: (1) light-addressable potentiometric sensors (LAPS) [22] and (2) pH sensors as (H^+ -) ion-sensitive field effect transistor (ISFET) [23, 24]. Both types of sensors provide output signals relative to a reference electrode. The LAPS was complemented by platinum electrodes (coated with a Nafion membrane) incorporated in the fluidic

head (plunger) for analysing oxygen. Additional platinum electrodes coated with the specific oxidase were incorporated to detect glucose and lactate [25].

MEMS – A New Generation of Miniaturized Integrated Devices

MEMS – or micro-electromechanical systems – are functional devices of mesoscopic size (up to millimetre range) that are assembled, most frequently by use of lithographic methods, from components of 1–100 μm size. They usually consist of central mechanical units, as flow channels and mixing chambers, combined with a central electronic unit for data processing, as a microchip. At this scale, microfluidics and the related problems of wetting and undesired electrostatics dominate. Only after these typical challenges are met, a functional unit is readily obtained.

These micromachines may be the basis for a lab-on-a-chip for external use as μTAS (micro-total analysis systems), but they are as well potential candidates as implants for continuous monitoring of physiological properties or for controlled drug administration.

Further Reading:

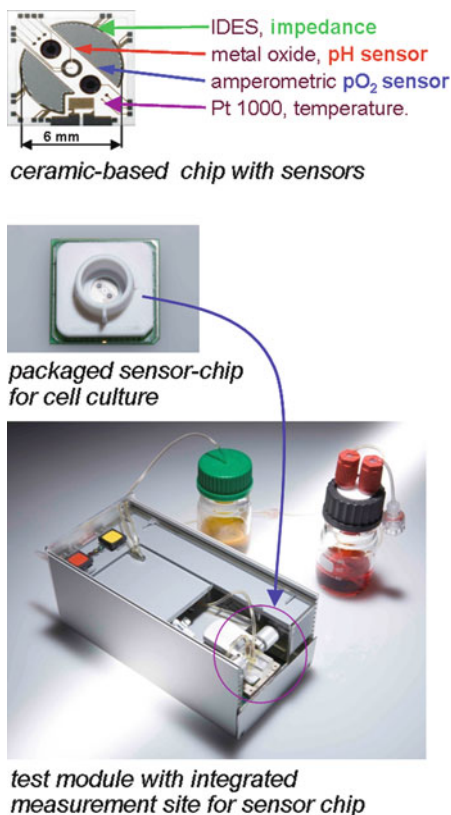
Lyshchinski SE (2006) MEMS and NEMS: systems, devices, and structures. CRC Press

Added by the editors

Wolf and co-workers [26] combined the ISFET pH sensor with an additional platinum electrode for sensing O_2 on the surface of the same silicon chip. Alternatively, Clark-like planar amperometric electrodes operating at a potential of -600 mV are being used [27]. Temperature control is obtained by measuring the forward voltage of a p/n-junction at constant current or the resistance of a platinum resistor integrated on the chip. Furthermore, interdigital electrode structures (IDES) are integrated on the chip for impedance measurements of cells and tissues (see Chapter 11). The size of these electrodes on the chip is in the range of 3–50 μm in width. Several different layouts combining these sensors on a single chip have been developed both on silicon and on glass basis [27, 28]. In each case, the cells grow in the vicinity of or in contact with the sensors.

These chips are packaged into a small culture containment (well) leaving a surface of about 38.5 mm^2 (7 mm diameter) for the cell culture [27]. The chips are sterilized, and approximately $4\text{--}10 \times 10^4$ cells in culture medium are seeded in to the well. After a pre-cultivation under standard conditions, this cell culture chip is placed into a custom-designed apparatus for measurements. A fluidic head has two channels connected to tubing for the addition and removal of culture medium; it is immersed into the culture medium and fits tightly within the well. The defined fitting creates a cultivation chamber with a volume of about 7 μl . The medium is transported to the culture at regular intervals via a peristaltic pump. In a typical protocol, the pump is on for 3 min and off for 7 min, i.e. one interval has 10 min. The signals obtained during the off-phase indicate the changes in the

Fig. 10.3 Example of a multiparametric ceramic chip with electric sensors (BioChipC) incorporated into a culture well. This chip, harbouring a cell culture, is placed into a test module and connected with a fluidic head for medium supply. The test module is connected to a PC for control of the fluidic and data acquisition. (Wiest et al. [29]; www.cellasys.de).



pH and O_2 concentrations produced by the cellular metabolism. The test apparatus can monitor the signals from the different sensors from up to six chips in parallel.

Instead of silicon, ceramics or glass can serve as alternative substrates, and the sensors are here produced in *thin film technology*. Glass has the advantage of microscope access to the probes. While Clark-like oxygen sensors are compatible with this technology, metal oxides, such as ruthenium oxide, were developed and serve as new types of pH sensors on this material. A recent ceramic-based chip design is shown in Fig. 10.3. This chip has similar dimensions as the silicon chip, but each is operated in a separate test module with specially adapted electronics [29]. Presently, it is possible to operate six such modules in parallel.

10.4.3 Opto-chemical Sensors

Another approach to detect changes in pH and oxygen is the use of *opto-chemical sensors*. These are organic fluorescent dyes, which change their luminescence after excitation depending on the partial pressure of oxygen or the pH in the

medium [30]. The pH sensor is a fluorescein derivative immobilized in a polymer, while the oxygen sensor is a luminescent probe based on a platinum(II)-porphyrine-derivative incorporated in hydrophobic particles. The read-out occurs by optic fibres. Presently, two test platforms are available for monitoring medium acidification and O₂ depletion by cell cultured in multi-well plates; they differ mainly in their fluidic and monitoring set-up.

In a custom 24-well plate with funnel-like wells, cells can be cultured in a conventional way. A special lid with inserts fitting into the culture medium of each well is placed on the plate. The insert (biocartridge) fits into the narrow part of the well just above the culture surface and leaves a sealed volume of 7 μ l culture medium for measurements, while the bulk of the medium is excluded. Along each biocartridge are four attached injections ports, which allow for the addition of pharmacological agents, toxins, etc. at prescribed times. At the immersed bottom side of this insert are a pH- and a pO₂-sensitive fluorescent sensors, which are monitored by optic fibres positioned in a sleeve from the top [31]; www.seahorsebio.com). For measurements, the plate is set into a test apparatus which is equipped with process control software. After a defined period of measurement, usually in the range of minutes, the inserts are lifted, thereby allowing the bulk medium to mix with the conditioned medium. This mixing can be repeated several times until the conditioned medium needs to be completely exchanged. The plate can be returned to an incubator after the measurement. The monitored signals are converted and expressed as extracellular acidification rate (ECAR) and oxygen consumption rate (OCR).

In another development of a 24-well set-up, each well is accompanied by two smaller chambers which at the bottom are connected by a small channel to the central well (Fig. 10.4a) [32]. In the central well, placed on a glass-based chip, are fluorescent sensors for pH and pO₂ as well as IDES for impedance measurements. (For the application of impedance measurements see Chapter 11.) Cells thus grow in the vicinity of these sensors. The lid of the plate has inserts which confine the volume of each culture chamber in the central well to 23 μ l. The side chambers contain a reservoir of culture medium in contact with the central well. The measured medium is exchanged by a robot pipetting system, which removes depleted medium from one side chamber and adds fresh medium into the other, which results in medium slowly flowing through the central chamber. This pipetting system also allows changing the culture medium conditions as well as adding and removing pharmacological agents, toxins, stimulants, etc. without removing the multi-well plate from the measurement platform. Since the sensors are at the bottom, the optic fibres are placed beneath the plate. With the cells receiving fresh medium periodically, they can be monitored continuously for several days.

There is also the option to move a custom-made microscope (Fig. 10.4b) aligned beneath the plate for imaging the cells in each well during the course of the experiment. In this case, the read-out of the optical sensors is carried out via the microscope optics. Moreover, this option allows the simultaneous documentation of the cell morphology.

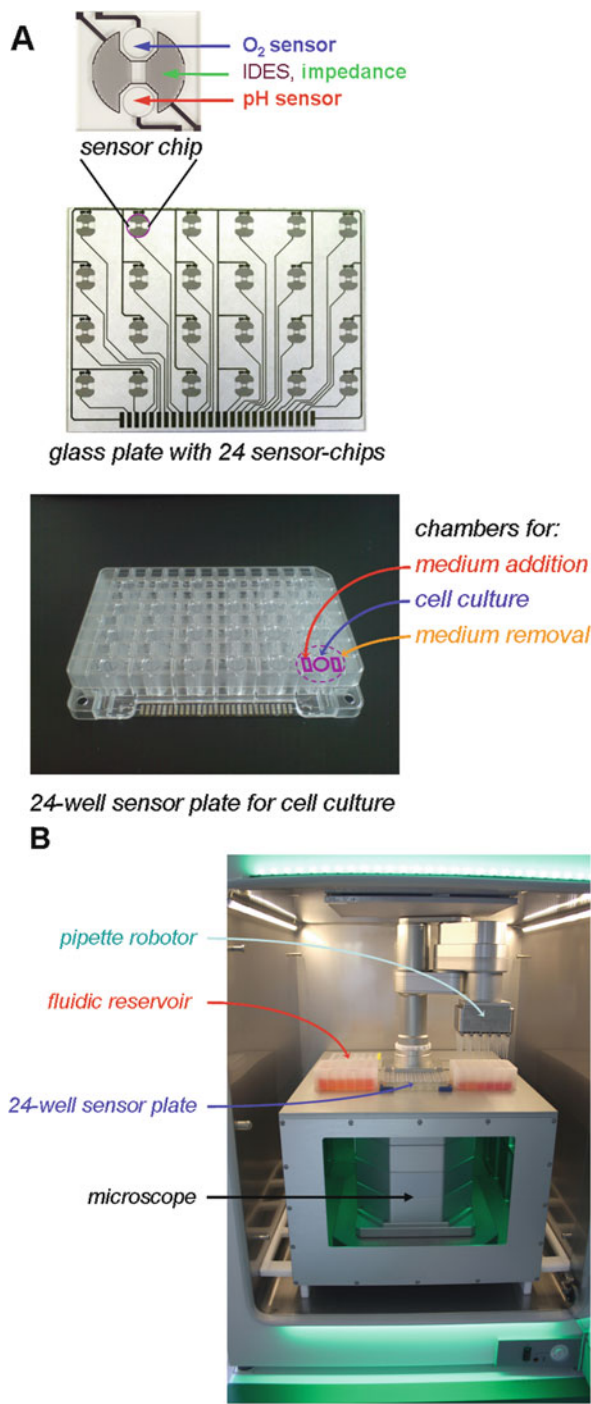


Fig. 10.4 (continued)

10.4.4 Evaluation of Measurements and Possible Interpretations

An essential part of these multiparametric test systems is the software for transforming the monitored electronic signals into terms of metabolic rates. In principle, the rates of extracellular acidification and O_2 consumption are calculated from changes in the signal amplitude obtained for a defined time in each interval where the medium was not exchanged (Fig. 10.5). For calibrated sensors, the signals are converted to values of pH and of oxygen content (% saturation).

The *rate of extracellular acidification* will reflect to a large part the rate of glycolysis. Under anaerobic conditions, acidification is mainly due to the production of lactic acid from pyruvate. However, under aerobic control, pyruvate also enters the tricarboxylic acid cycle (Krebs cycle), where other acids will be produced, e.g. carbonic acid from CO_2 (see Fig. 10.1) [2].

The term *rate of O_2 consumption* is an indicator of cellular respiration. Inhibitors of the respiratory chain have been shown to markedly attenuate oxygen consumption [31]. But it should be kept in mind that the formation of radical oxygen species (ROS) may also contribute to the balance of oxygen consumption

10.4.5 Some Applications

These different sensor chip test systems are serving an increasing number of investigations in the biomedical field, as yet mainly in cytotoxicity testing and tumour biology. A few applications which have been published will be described here.

For *cytotoxicity testing*, LAPS have been used to test the response of the liver cell line HepG2 to inflammatory cytokines [33]. The measurements showed that there was 20 and 60% *increase* in the rate of acidification within 30 min after the addition of interleukin-1 (IL-1) and oncostatin M, respectively. With this technology, it was also possible to monitor the recovery phase upon removal of the cytokines as well as the response following the addition of another cytokine. In a different study, multiparametric silicon chips were used to measure the effect of a series of inorganic compounds in mouse fibroblast (BALBc 3T3) cultures [34]. Over a course of 26 h, sodium arsenite, cadmium chloride, and *cis*-platinum each inhibited O_2 consumption to a greater extent than the acidification rate, albeit with different kinetics. Upon removal of these toxins, there was no cell recovery. Together these exemplary reports, besides providing information on the dynamics of metabolic inhibitions, illustrate the advantages of a test system with integrated fluidics which permit to

◀ **Fig. 10.4** A complete multiparametric chip test system based on opto-chemical sensors. (a) The 24-well glass plate and its layout. Each of the 24-wells has an integrated sensor chip and two small medium containers aside each well for medium exchange. (b) The measuring platform. The plate is set into a test apparatus which is constituted of the pipetting robot, the tray for the sensor chip plate and the respective plates for providing medium and test solutions as well as plates for disposal. The read-out is performed via a custom microscope. Not shown are control units and the monitor. (Lob et al. [32]; www.hp-med.com).

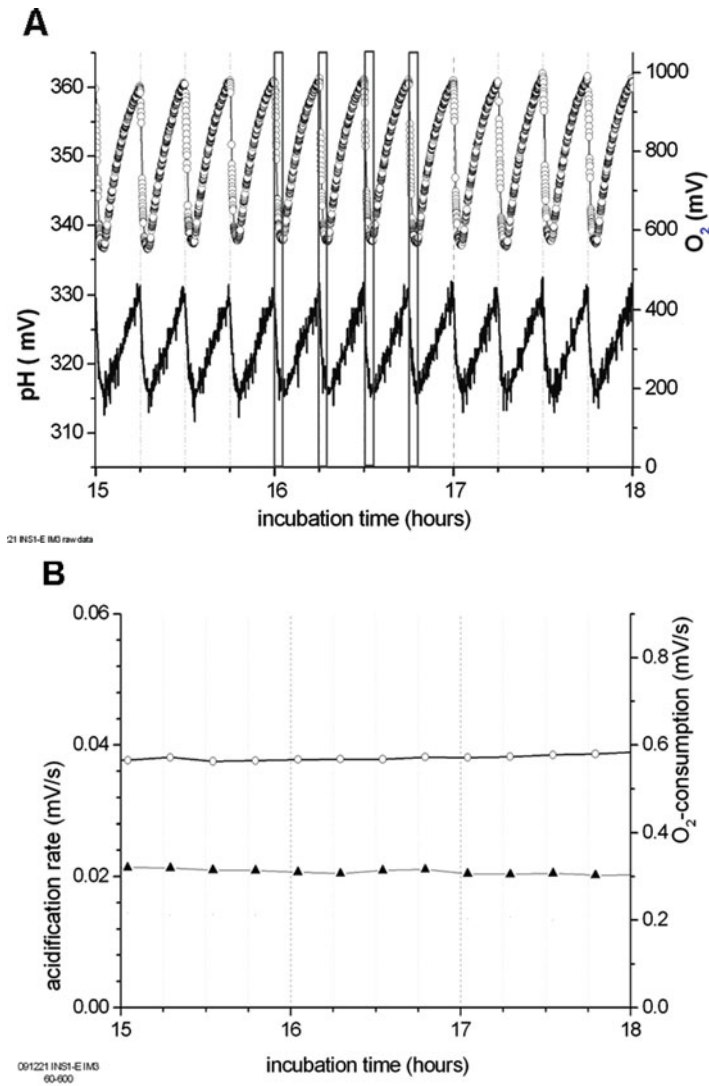


Fig. 10.5 Example of signal evaluation of sensor chip measurements monitoring changes in extracellular pH and oxygen consumption. In this example, INS-1E cells were cultivated on electric sensor chips (BioChipC). During the measurement, medium was exchanged for 3 min (a, columns) in each of the 15 min intervals. The monitored signals from each slope during the stationary incubation phase (a) are calculated as rates of change (b). (Otto, A. and Bergmann, B., unpublished data).

add and remove drugs without interference of the cell culture set-up. The beauty of this test system is therefore that the metabolic response of an individual cell probe can be monitored with specified changes in the experimental protocol.

Multiparametric silicon sensor chips have been also implemented in several studies on *tumour cell metabolism and chemosensitivity*. Using different tumour cell

lines, the temporal development of growth inhibition by different drugs, such as chloroacetaldehyde, cytochalasin B, and doxorubicin have been monitored [35]. Studying the divergent effects of chloroacetaldehyde on the metabolism of the colon cancer cell line LS174T, it could be shown with the sensor chips that the rates of O_2 consumption and, slightly retarded, also of extracellular acidification were attenuated over a period of 24 h. Various biochemical assays were used to complement these measurements at various time points after drug addition. Using the fluorescent dye JC-1, a rapid depolarization of mitochondria was observed within about 30 min after drug addition; this correlated well with the attenuated rate of O_2 consumption. In contrast, during the first 3 h there was a transient increase in intracellular ATP levels, as quantified with the luciferase bioluminescence assay [36]. Contrary to the expectations, cellular ATP content thus may not be directly related to the rates of extracellular acidification and O_2 consumption.

Extracellular acidification and O_2 consumption by a cell probe may also have divergent dynamics. As a marked example, using cytochalasin B, an actin-depolymerizing drug which also inhibits glucose uptake, the rate of acidification was virtually immediately reduced, while O_2 consumption increased; and this effect was reversed by drug removal.

The *fluorescent sensor system* is being likewise used for investigations on energy metabolism in a variety of tumour cells. Analysis of the effect of several glycolytic and mitochondrial inhibitors, e.g. 2-deoxy-glucose, 2,4-dinitrophenol, and rotenone, also showed how acidification and O_2 consumption are differently affected [31]. When looking at the genetic control for the regulation of energy metabolism, those tumour cells which expressed the intact tumour suppressor gene *PTEN* had lower rates of acidification and O_2 consumption than those cells lacking the expression of this gene; its silencing resulted in increased glucose consumption and enhanced proliferation [37]. Thus, the oncogenic status of these cells could be correlated with their metabolic activity.

On the basis that mitochondrial activity is essential for *insulin secretion*, the fluorescent sensor test system has been used to measure the respiratory activity in the β -cell line INS-1 [38]. This study analysed mitochondrial dysfunction as manifested in a defect in the mitochondrial fission machinery: not only did this effect result in a reduced O_2 consumption rate but also in impaired insulin secretion.

Taken together, these different applications illustrate the broad scope of scientific investigations to which multiparametric sensor chips for measuring metabolic activity in real time can provide valuable information on living cells.

10.5 What Can Sensor-Based Method Contribute to Systems Biology of Islets and β -Cells?

The multiparametric sensor chip systems, by virtue of monitoring both extracellular acidification and oxygen consumption of cultured cells and tissue in real time, are predestined to serve investigations on the metabolic and functional activities of β -cells and tissues. These test systems can provide three types of information from the same probe: (1) the rates of acidification and oxygen consumption, (2) their

relationship to each other, and (3) the temporal dynamics of metabolic changes (response time, kinetics, reversal of effect, etc.).

These are relevant data of a biological response which complement the “snapshot” data (i.e. a specific time point only) obtained with genomics, proteomics, and metabolomics. By contributing the dynamic aspects, monitoring parameters of energy metabolism in real time is a top-down approach, which should meet the bottom-up approach, called for in [Chapter 1](#), when the same types of cellular probes and the same protocols are used.

Acknowledgements In this chapter I have described some multiparametric sensor chip platforms developed by Professor B. Wolf and co-workers at the Heinz Nixdorf Chair for Medical Electronics, Technische Universität München. This work over many years has been financially supported by the Heinz Nixdorf-Stiftung, the German Ministry of Education and Research (BMBF), the Bavarian Research Foundation (Bayersche Forschungsförderung, BFS), the German Research Council (Deutsche Forschungsgemeinschaft, DFG), as well as industrial partners. I thank my colleagues for fruitful collaborations, with special thanks to Drs. B. Gleich, H. Grothe, and J. Wiest, to B. Becker and to Prof. B. Wolf for providing images and critical reading of the manuscript.

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Chapter 11

Bioimpedance Spectroscopy

Beate Klösgen, Christine Rümenapp, and Bernhard Gleich

Abstract In the context of biology, electrical phenomena are usually identified with ionic currents through protein channels, for example as they are postulated during nerve signalling (Andersen et al. *Prog Neurobiol* 88(2):104–113, 2009; Sakmann and Neher *Annu Rev Physiol* 46:455–472, 1984). However, there are more electrical phenomena that play a significant role in biological systems, namely those arising from polarization effects (Grimnes *Bioimpedance and Bioelectricity*, 2008; Polk *Handbook of Biological Effects of Electromagnetic Fields* 1996). Local inhomogeneities in charge distributions give rise to the formation of permanent molecular dipoles as in uncharged molecules such as (hydration) water, or due to the polar groups in proteins and lipid molecules. Non-permanent electrical dipoles can originate from the presence of ions in solution. Structured distributions of counter-ions at all polar interfaces, for example along the surface of proteins and especially along the polar membrane interfaces of cells, cause the formation of Stern and Helmholtz layers. All non-uniform distributions of charges and dipoles initiate and modify internal local electrical fields. Moreover, the application of external fields causes relaxation processes with characteristic contributions to the frequency-dependent complex dielectric constant. These dipolar relaxations were initially described by Debye (*Polare Molekeln* 1929). They are the basis of impedance spectroscopy (K' Owino and Sadik *Electroanalysis* 17(23):2101–2113, 2005; Schwan *Adv-Biol-and-Med-Phys* (5):147–209, 1957; Schwan et al. *J Phys Chem* 66(12):2626–2635, 1962). The dispersion and the related adsorption contributions of the dielectric spectrum yield the dipole-specific relaxation frequencies and also give information about the dipole density. Redistributions of dipoles, binding events and changes in local viscosity will all appear as modifications of the signal amplitude and in system-specific frequency shifts. These changes in the measured spectra can be used in a variety of technical devices, for example for biosensing as well as for monitoring the properties of cells in cell culture.

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Keywords Impedance spectroscopy · Dielectric relaxation spectroscopy · Lab-on-a-chip · Biosensor

11.1 Introduction

Non-invasive techniques for the investigation of complex processes such as the monitoring of chemical reactions and changes of composition and shapes are considered attractive tools in many areas of science and engineering and especially for medical applications. Among the early options, the in vitro and even in vivo investigation of electrical properties of materials was identified as a potential tool for non-invasive methods: apparatus were seemingly simple and ready at hand, consisting essentially of power supplies and electrodes. The acquisition of electroencephalograms or electrocardiograms is nowadays standard in medical diagnosis. Still, electrodes may be considered in a more general way as a tool either for the injection of currents into biomaterial with the goal of measuring impedance responses or for the application of fields to investigate dielectric polarization responses. These two apparently different methods address two aspects of the same phenomenon, namely the frequency-dependent complex dielectric constant that is a material property of specific constituents, composition and geometry and micro-surroundings.

At zero field frequency, in the case of a so-called direct current (DC) situation, the electrical properties of the material are essentially represented by the Ohm resistance with its specific conductivity due to mobile charges. The dielectric properties are hidden in the presence of the passive capacitance due to interfacial charge accumulation and dipole alignment within the dielectricum. They only show up during the switching-on or switching-off process (see Section 11.2.5.1) but do not contribute to the steady current (DC).

At very high frequency, in the case of an AC situation, the dielectric properties are reflected in the complex refractive index, consisting of a dispersion (real) and an absorption (imaginary) part. The relative impact of conductivity versus dipole orientation is shifted as frequency increases. Therefore, DC resistance is replaced by AC impedance that takes both contributions into account. Results may be depicted in Cole–Cole plots [23, 91, 92] of the impedance or in plots of the two components of the dielectric constant as a function of frequency [28]. The methods are still under development and refinement, especially with the goals of miniaturization and of enhanced resolution, but can now be considered as established [8, 51, 70, 109].

11.2 Theoretical Background of Bioimpedance Spectroscopy

11.2.1 General Considerations

Isolated charges are rare in nature under equilibrium conditions: each charge usually has a countercharge close by so that matter seen from the outside is essentially

electrically neutral. Only within small volumes, differences in electronegativity lead to local charge inhomogeneities as for example the formation of ions upon the dissolution of salts in aqueous solution or the formation of polar regions in molecules (partial charges).

Charges are the sources of fields. Their separation results in electric field gradients, and thus in differences of electric potential. Negative charges will be drawn to higher potential, and positive ones to lower potential. This is the origin of electric currents, and of the formation of shells of counter-ions around dissolved ions of opposite charge.

It will be shown that in principle all these phenomena may be integrated into the concept of dipoles (or multipoles). The ionic currents in electrolyte solutions are in a sense always dual currents where an electric potential difference $\Delta\Phi$ causes a motion of cations in one direction and a related flow of anions in the opposite direction. The fractional respective contributions to the total current are described by ionic transfer numbers, and the solution is polarized as long as the field is applied. The total current i follows Ohm's law:

$$\begin{aligned} i &= \frac{\Delta\Phi}{R} = \Delta\Phi \cdot G \\ &= \Delta\Phi \cdot \sigma \cdot g \end{aligned} \quad (11.1)$$

where R is the resistance and its inverse G the conductance.

The conductance can be broken down to a material-specific conductivity σ and a geometrical factor g (usually $g = \frac{A}{l}$, for the case of a current between two parallel metallic plates of area A and distance l that are connected to a power supply).

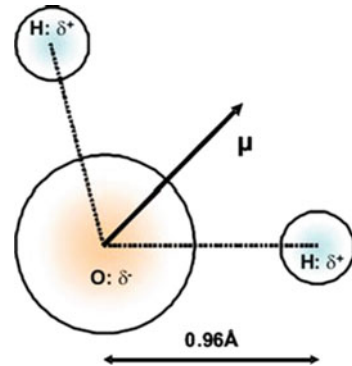
However, with increasing frequency ions become restricted to move due to inertia but rather respond to the potential by re-orientation of their dipole moments: the ionic conductivity σ drops from the steady state value σ_0 . In this case (details see below), the system is better described by the complex dielectric constant

$$\varepsilon = \varepsilon' + i\varepsilon'' \quad (11.2)$$

11.2.2 Dipoles and Polarization

Dipoles are abundant in nature: they appear as soon as the centres of negative and positive charge distributions do not coincide. Their strength is described by the dipole moment $\underline{\mu}$ (the vector character of the dipole moment is abandoned here and μ is adopted for ease of discussion as scalar dipole moment). Upon bond formation among atomic partners of very different electronegativities, molecules with permanent dipole moments are formed [28]. The most prominent example is water [22] (see Fig. 11.1), not only because of its large quantity but also because of its role as a solvent for many chemical reactions and especially its role in biosystems [7, 22, 58, 97, 100, 114, 121]. External electric fields may enhance permanent dipole moments [99, 117, 123, 129], and initially nonpolar material may acquire an induced non-permanent dipole moment [10, 73, 116, 117].

Fig. 11.1 Schematics of water molecule H_2O as an example for molecular dipole due to partial charges δ^+ and δ^- . Dipole moment: $\mu = 1.85 \text{ D}$, angle suspended between oxygen–hydrogen bonds: 104.5° .



Dipoles interact with each other. Molecules with permanent molecular dipole moments tend to align and to develop ordered structures with a spontaneous macroscopic polarization $\underline{P} = \sum_i \underline{\mu}_i$ as in liquid crystals [25]. The dipole characteristic of fluctuating electron density even gives rise to the van der Waals interaction [83]. The degree of structure formation is hampered by temperature as thermal energy induces fluctuations into the system of interacting dipoles and thus prevents the formation of perfect dipole crystals at all realistic temperatures. As a result, the spontaneous polarization obtained from dipole interactions is permanent only below a sufficiently low temperature (called Curie temperature) and then the material is ferroelectric [5, 6, 54, 60, 81, 99].

Still, some local order may spontaneously arise due to dipole interactions, for example along charged interfaces. This is the case when electrodes are in contact with electrolyte solutions. Here, an electrochemical reaction with electron charge transfer results in the built up of an interfacial Nernst potential [41] (see also Galvani potential). A different case is the interface between cell surfaces (biomembranes) and adjacent liquid. Most cell membranes carry a net negative surface charge as there are only neutral or negatively charged lipids in nature. Moreover, most proteins are as well slightly negatively charged under physiological conditions ($\text{pH} \sim 7.4$). Therefore, biointerfaces are typically negatively charged and exhibit a surface electric potential Φ_{surface} that exponentially decays into the equilibrium potential of the bulk liquid with a characteristic constant λ_D , the Debye length

$$\Phi_{\text{surface}}(x) = \Phi_0 \cdot e^{-\lambda_D x} \quad (11.3)$$

where x signifies the distance from the cell surface with potential Φ_0 . If the cell was not immersed in electrolyte solution, the surface potential would decay infinitely slowly. The presence of charges and polarizable components in the electrolyte solutions around a charged surface (ions, cell membranes, electrodes, etc.) causes a faster decay of the electric potential. This is evident in a smaller Debye length – the surface charge is said to be screened.

The value of the Debye length λ_D depends on the ionic strength I of the electrolyte solution around the biological cell or tissue, the dielectric constant ϵ' of the solution and the temperature T so that for dilute salt solutions and in the limits of the Debye–Hückel theory [51, 97],

$$\lambda_D = \sqrt{\frac{\epsilon_0 \epsilon' \cdot kT}{2N_A e_0^2 \cdot I}} \quad (11.4)$$

with $I = \frac{1}{2} \sum_i c_i z_i^2$ for the ionic strength.

c_i and z_i are the molar concentration and charge number, respectively, of the i^{th} type of ions in the electrolyte solution; ϵ_0 the vacuum electric permittivity; N_A the Avogadro constant; e_0 the elementary charge and k the Boltzmann constant. All ions present in the electrolyte solution contribute to the value of I . For example, Eq. (11.4) yields values of $\lambda_D \approx 1$ nm for 100 mmol NaCl but $\lambda_D \approx 0.6$ nm for 100 mmol CaCl_2 , because of the higher ionic strength due to the divalent Ca^{2+} , and $\lambda_D \approx 0.8$ nm for 140 mmol NaCl ($\sim$ physiological osmolarity). Equation (11.4) is an approximation for dilute ionic solutions and it will, for many ions, fail in the range of physiologically relevant concentrations. In that case, either semi-empirical corrections must be applied or the effective values of λ_D must be determined experimentally by measuring the zeta potential for ion concentrations above 100 mmol [51].

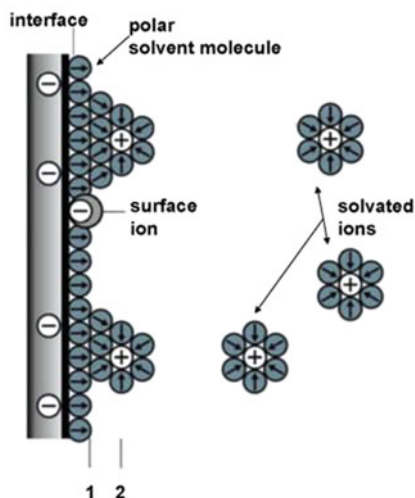
11.2.3 Electric Dipole Layers

Following the Gouy–Chapman theory, the reason for the decay of the surface potential is the formation of interfacial dipolar layers whenever the interface is immersed in electrolyte solution (see Fig. 11.2). In biological systems, the dipoles of the solvent water will wet the polar interface by orienting themselves appropriately: the end of the water dipole with the positive partial charge will on average be closer to a negatively charged interface than the opposite end. A first hydration layer forms. At the same time, the layer potential will decrease proportionally to $\sqrt{\epsilon'}$, where ϵ' is the zero frequency real part of the dielectric constant of water (~ 81). ϵ' is also called electric permittivity.

Through the first hydration layer a dielectric polarization is generated that decreases the electric field in the solvent as compared to the value it would have had without the dielectric polarization. The next water layer has the same orientation but is less ordered due to the higher distance from the charged interface. Proceeding further towards the bulk water phase, the orientation of the dipoles gets more and more diffuse until finally no net polarization persists and the water dipoles are uniformly oriented on average: this condition is reached for $\lambda_D x \cong 8$.

In electrolyte solutions when there are ions dissolved in water, each ion carries its own hydration shell (see Fig. 11.2) and can be envisaged as a dissolved dipole by its own. Thus, another type of polarization will additionally be caused by a redistribution of the ions along polar interfaces, causing the formation of Stern

Fig. 11.2 Formation of interfacial layers. Solvent shells of decaying polarization due to negative interfacial charges. Accumulation of positively charged ions in layers close to the interface. Bulk ions in hydration shell. 1, inner Helmholtz layer; 2, outer Helmholtz layer.



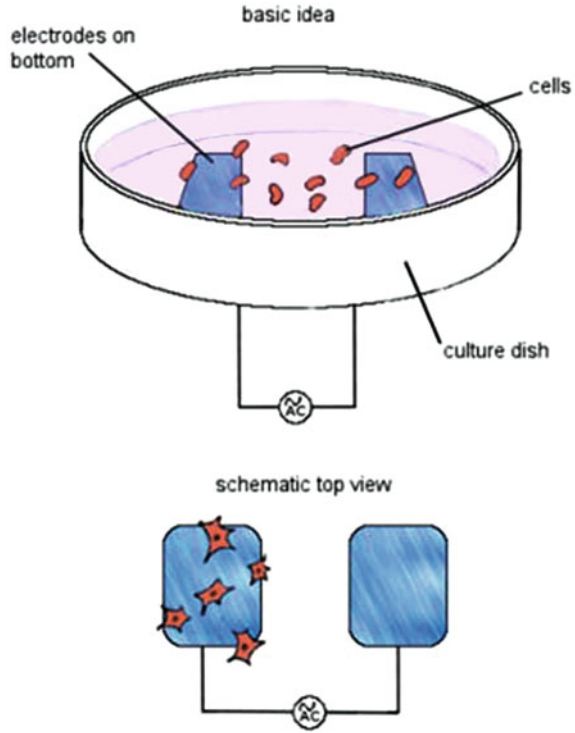
and Helmholtz layers [12, 19, 69, 93, 97, 100, 138]. In the example of Fig. 11.2, positively charged ions are preferentially dragged towards the negatively charged interface whereas their negatively charged counter-ions are repelled. Thus, ionic double layers are formed (Helmholtz layers), again with decreasing order as the distance from the interface increases. It might even occur that ions partially slip off their hydration shell and get specifically adsorbed to form a well-defined Stern layer (not shown here). The potential drops exponentially, its course being determined by the Debye length λ_D as approximated by Eq. (11.4), depending both on the ionic strength due to the electrolyte and on the dielectric constant of the solvent. Again, thermally induced fluctuations counteract the formation of such meta-stable dipole-driven structures and the layers become increasingly diffuse with increasing distance from the polarizing interface. Finally, at sufficient distance, the interfacial charge is totally screened and the electrolyte solution becomes a homogeneous liquid of uniformly distributed dissolved ions.

11.2.4 Effect of External Fields

11.2.4.1 Motion in External Fields

External electric fields may be applied to any kind of sample by firmly contacting metallic electrodes to it (see Fig. 11.3, to be discussed further later on) and then connecting the electrodes to a power supply. The presence of the external field will influence and enhance the formation of dipoles, and will induce a macroscopic polarization (see below). The electric forces on charges may cause electro-migration or electro-rotation [39, 47, 107]. Electro-migration may be used to position and guide colloidal particles, like small drug crystals, vesicles or whole cells [124].

Fig. 11.3 Sketch of set-up for performing impedance measurements on a cell culture under the fluorescence microscope. Cells are grown on an agar gel in a Petri dish in proper buffer conditions. Metallic electrodes are attached to the bottom of the dish and attached to an AC power supply and serve to apply various types of electric fields. The top view exhibits that some cells migrated to one of the electrodes under the influence of an applied field and firmly attached to it.



Alternatively, in electrophoresis, it may serve for analytical purposes, making use of the electrophoretic mobility u of charged particles

$$u = \frac{v}{E} \quad (11.5)$$

where

$$E = \frac{\Delta\Phi_{\text{applied}}}{d} \quad (11.6)$$

is the electric field due to the applied external potential difference (voltage) $\Delta\Phi_{\text{applied}}$ between the electrodes of spacing d , and v is the so-called drift speed of the colloidal particles. The drift speed may be observed directly when watching the motion of the particles via optical microscopy. It depends on the effective charge of the moving particle ($z_i \cdot e_0$) as well as on the friction the object experiences when moving in its surroundings

$$u = \frac{z_i \cdot e_0}{6\pi \cdot \eta \cdot r}. \quad (11.7)$$

Here, η is the local viscosity which the mobile particle of radius r experiences from its surrounding medium (usually, the buffer). However, such a simplified system is not realistic since the particle carries its double layer (Fig. 11.2). In a more precise approach, one has to consider the surface charge of the particle with the resulting polarization of the adjacent Helmholtz layers and the contribution of the diffuse layer to the viscosity. Two extreme cases can be distinguished here, namely the one where the particle is small compared to its Debye length ($\lambda_D \cdot r = 1$, small particle with relatively thick double layer) and the other one, where a very big particle is surrounded by a relatively thin double layer ($\lambda_D r \rightarrow \infty$). Both are described by the theory of Hückel and Smoluchowski, respectively:

$$u = \frac{2\varepsilon_0\varepsilon'\zeta}{3\eta} \text{ for } \lambda_D \cdot r = 1 \quad (11.8)$$

$$u = \frac{\varepsilon_0\varepsilon'\zeta}{\eta} \text{ for } \lambda_D \cdot r \rightarrow \infty \quad (11.9)$$

The term ζ in these equations is the so-called zeta potential [83, 91, 93] that signifies the value of the surface potential at the location of the slipping plane that separates the surface-bound fluid (Helmholtz layers) and the freely mobile bulk fluid. The value and position of ζ can be determined experimentally.

11.2.4.2 Polarization in Electric Fields

External fields will apply forces to charged particles and dipoles, and they induce dipole moments to initially nonpolar systems and as well enhance initial dipole moments. As another effect, external fields may partially compensate the disturbing effect of thermal fluctuations. Therefore, the dipole–dipole interactions are strengthened compared to the situation where the external field is zero.

The external electric field will introduce a preferred direction into a system. It will apply an external force to the dipole moments. Thus, it will re-orient all dipole moments towards its direction if the viscosity is low enough, and it will stabilize the orientation of dipole moments in the direction of the field against thermal fluctuations. As a response to the external field, an additional polarization is induced into the dipole system that may overcome the spontaneous one by far. The dependency of the polarization on a steady applied field (DC field) is described by the Langevin–Debye function that accounts for the interplay of thermal disturbance (entropy) and electric organization (enthalpy).

11.2.4.3 Oscillating (AC) Fields

In general, the field must not be constant but may oscillate with a frequency f . The response of a system then depends on the system dynamics because the different possible processes are not equally compatible with the exciting frequency. The general course of the dielectric spectrum is schematically shown in Fig. 11.4.

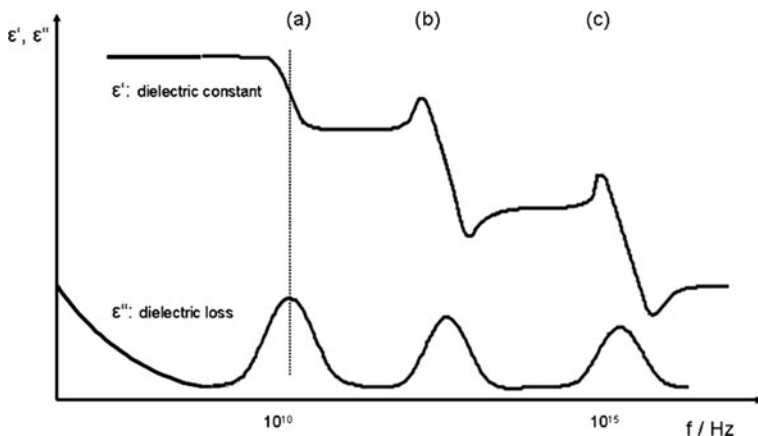


Fig. 11.4 Principal course of dielectric spectra, consisting of dispersion (ϵ') and absorption (ϵ'') loss spectrum. (a) dipole relaxations, (b) atomic vibrations and (c) electronic transitions. Each individual process contributes by a step to the total course of the dielectric spectrum. The initial decay on the very low-frequency slope of ϵ'' arises from conductivity contributions. The initial constant contribution in ϵ' is the steady-state dielectric constant.

11.2.5 Dielectric Spectrum

The dielectric spectrum, as schematically shown in Fig. 11.4, exhibits two curves, namely the dispersion curve $\epsilon'(f)$ and the loss/absorption curve $\epsilon''(f)$. Depending on the frequency range, two characteristic courses of the curves can be distinguished, attributed to relaxation (a) and to resonance processes ((b) and (c)). Of course, each system exhibits a whole series of these transitions.

The resonances are observed in the frequency range above 1 GHz ((b) and (c)) and occur when quantum mechanical processes take place: the (b) region is typical for transitions in molecular excitations (rotations, vibrations) and the (c) region accounts for the electronic transitions. Typical for resonance processes, the loss curve shows absorption peaks of Lorentzian shape with a natural line width Δf_L (width of the line at half maximum intensity) and an ideal decay/transition lifetime $\tau_{L0} = \frac{\Delta f_L}{2\pi}$ at low temperature and dilution. This ideal condition is almost never met as the excited particles cannot be prevented to exchange momentum when they bump into one another due to their thermal motion. This causes the so-called Doppler broadening of the natural line width such that $\tau_{LD} > \tau_{L0}$. The absorption peak is still very sharp, and its shape is still of Lorentzian type. The related dispersion curve exhibits abnormal dispersion with a negative slope in the region of an absorption peak and, in between two different resonance peaks, normal dispersion is found as it is known from optics, with the refractive index n ($n = \sqrt{\epsilon_0 \epsilon'}$) increasing with frequency.

The course of the dispersion and loss curves is totally different in the low-frequency range (a): first, the absorption peaks are much broader compared to the

resonance peaks, exhibiting a Debye shape. The dispersion curves start with a constant value of ε' followed by a smooth continuous drop $\Delta\varepsilon'_j$ for each distinct relaxation event that takes place (see Fig. 11.16). This part of the dielectric spectrum is the range of the dipole relaxations.

11.2.5.1 Properties of the Dipole Relaxation Spectrum

Dipole relaxations occur in the frequency range Hz to GHz. They are characterized by a typical course of dispersion ($\varepsilon'(\omega)$) and absorption ($\varepsilon''(\omega)$) curves that are connected by the Kramers–Kronig relations [48, 64, 65, 68]. The course of $\varepsilon'(f)$ and $\varepsilon''(f)$ was first described by Debye [28] as

$$\varepsilon'(f) = \varepsilon_\infty + \sum_j \Delta\varepsilon_j \frac{1}{1 + (f/f_j)^2} \quad (\text{dispersion, permittivity}) \quad (11.10)$$

$$\varepsilon''(f) = \Delta\varepsilon_j \frac{(f/f_j)}{1 + (f/f_j)^2} \quad (\text{absorption, loss}). \quad (11.11)$$

Here, f is the frequency of the exciting field (electromagnetic wave) and f_j the relaxation frequency of the j th dipole system. Each type of dipole (dipole system) contributes to a drop $\Delta\varepsilon'_j$ to the decay of ε' until the final value ε_∞ at infinitely high frequencies (optical range) is assumed.

In the purely Debye case, the course of ε' and ε'' is symmetric on $\ln(f)$ and the peak maximum of the loss curve (ε'') is found at a frequency where the dispersion curve (ε') exhibits a turning point (see Fig. 11.4). Dielectric relaxation processes can be modelled by a harmonic oscillator that is excited by the electric force of the field on the dipole moments, resulting in a characteristic response eigenfrequency for each type of responding dipole system. This frequency is called relaxation frequency f_0 , and it corresponds to a relaxation time τ_0 . The relaxation frequency is characteristic for the dipole system (e.g. the dipoles of bulk water) and coupled to the relaxation time by

$$f_j = 2\pi\omega_j = \frac{2\pi}{\tau_j} \quad (11.12)$$

At zero frequency (constant field), the system is characterized by its resistance R and its electrical capacitance C . $C = \varepsilon_0 \cdot \varepsilon'(f = 0) \cdot g$ is determined by the zero frequency dielectric constant $\varepsilon'(0)$ that describes the steady polarization obtained in a material filled capacitor with the according dielectric properties. g is a geometric factor, which in case of a parallel plate capacitor is $g = \frac{A}{d}$ with d being the distance between two metallic plates of area A . The dipoles present will acquire a constant polarization $P = \varepsilon_0 \varepsilon' E$. The presence of mobile charge carriers like ions in water will lead to a current $i = \frac{\Delta\Phi}{R} = \text{const}$ with an Ohm-type resistance R .

In the case of ions, R is given by the respective ionic conductivities following, e.g., the Debye–Hückel theory [97]. The presence of the direct current appears in the dielectric spectrum at the very low end with a decaying wing in ε'' . Apart from the

switching-on and switching-off processes, a steady situation with constant current and polarization develops.

As soon as time-dependent fields are applied, the situation changes: as discussed above, the response obtained now depends on the system dynamics. Most evident, the mobility of the ions is limited. Therefore, the direct current decreases to zero as frequency increases (see Fig. 11.4) – the hydrated ions can no longer move in the field. Still, dipole polarizations will occur up to the GHz range when the relaxation of liquid water (~ 19 GHz) sets the end of dipole relaxations (all other dipoles in electrolyte solutions are less mobile) and the spectrum continues with the resonances. This is first realized in the context of the switching-on and switching-off processes when both the current and the polarization response are observed to be delayed with respect to the field:

$$P(t) = P_{\max} \cdot e^{-\tau t} \text{ (switching-on process)} \quad (11.13)$$

$$P(t) = P_{\max} \cdot (1 - e^{-\tau t}) \text{ switching-off process} \quad (11.14)$$

In such a so-called time-domain experiment, the measured time course can be disintegrated into contributions of j characteristic exponential rates of typical relaxation times τ_j such that

$$P(t) = P_{\max} \cdot \sum_j e^{-\tau_j t} \text{ (switching-on process)} \quad (11.15)$$

$$P(t) = P_{\max} \cdot \left(1 - \sum_j e^{-\tau_j t} \right) \text{ (switching-off process)} \quad (11.16)$$

Each characteristic rate obtained signifies system properties that depend on the interaction between specific dipoles, the temperature and the viscous properties of the dipole surroundings.

11.3 Experimental Set-up

Since the times of Debye, the investigation of the frequency-dependent response of dipole systems became the basis of the emanating techniques of dielectric spectroscopy or electrochemical impedance spectroscopy [12, 19, 52, 58, 69, 85, 93, 96, 104, 109, 110, 112, 114, 118–120, 138]. The names essentially result from the different application communities: dielectric spectroscopy focuses on the spectroscopic aspect that considers the dipole relaxations as a peculiar part within the total dielectric spectrum (this starts with the classical dipole relaxations and continues into the resonance spectra of the high-frequency regions that are dominated by quantum mechanical effects). The term impedance spectroscopy on the other hand emphasizes the aspect of measurement and engineering, interpreting the relaxations as contributions to the complex electrical resistance (impedance), observed in

a system responding to the excitation of an external field from the mHz to the GHz radiofrequency range [111].

Equations (11.13), (11.14), (11.15), and (11.16) represent prescriptions to perform dielectric spectroscopy by measuring the build-up or decay of polarization as a function of time after an instantaneous change of the electric field (electric step function). Such experiments are called time-domain spectroscopy. The alternative are the so-called frequency-domain experiments that are based on measuring the response of a system as it is excited by an oscillating field of frequency f and subsequently scanning the frequency stepwise in the (whole) dipole relaxation range 0 Hz to GHz. The different options are shown in Fig. 11.5. The results obtained from either method are equivalent and may be interconverted by Fourier transformation.

The total macroscopic polarization of the system can be measured by electrode set-ups that compare the amplitudes and phase shifts of a transient or reflected wave with the initial field applied or by impedance set-ups that determine the value and phase shift of an electric current upon an applied voltage [8]. Both methods are equivalent and yield the field response that can be described by the complex dielectric constant $\varepsilon = \varepsilon'(\omega) + i\varepsilon''(\omega)$, with an absorption term ε'' and a dispersion term ε' .

In terms of electrical components, this time-dependent behaviour can be described by complex impedances Z_j as a combination of resistances R_j and

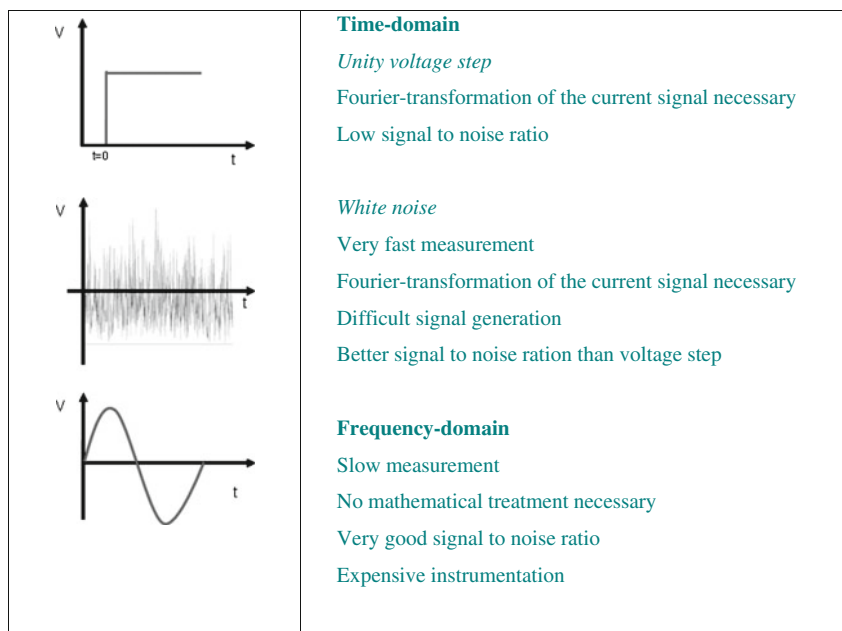
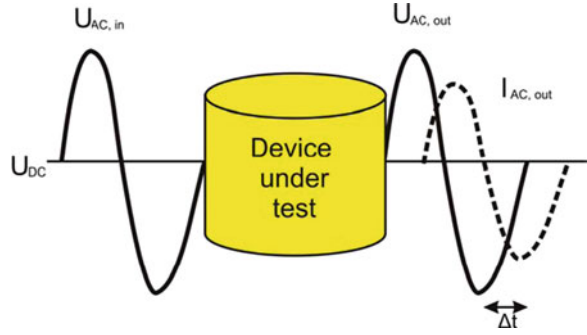


Fig. 11.5 Principal options for conducting frequency- or time-domain dielectric experiments. Excitation voltage as a function of time is either a step-function or a sinoidal wave. In the latter case, the acquisition of the full spectrum requires scanning all possible frequencies.

Fig. 11.6 Scheme of set-up for impedance spectroscopy



capacitors C_j . The principal set-up for such an experiment is sketched in Fig. 11.6.

An incident wave $U_{jAC,in} = U_0 \cdot e^{i(\omega t + \varphi_U)}$ with an amplitude U_0 , angular frequency ω and phase φ_U is applied to a sample. The outgoing wave $U_{jAC,out}$ and the measured current $I_{jAC,out} = I_0 \cdot e^{i(\omega t + \varphi_I)}$ are phase shifted by $\Delta\varphi = \varphi_U - \varphi_I$ as observed in a delay time Δt .

The complex impedance is then defined as

$$Z_i = \frac{U_{iAC,out}}{I_{iAC,in}} \quad (11.17)$$

and consists of a real part called resistance (coinciding with the value of R found under steady-state DC conditions) and an imaginary part called reactance.

The reactance accounts for the build-up of internal fields due to the exciting wave, in our case the polarization P as a response to the exciting field E . This term is responsible for the overall delay/phase shift.

The impedance Z is given as

$$Z = Z_R + Z_C = R - i \frac{1}{\omega C} \quad (11.18)$$

where Z_R and $Z_C = \frac{1}{i\omega C}$ are the resistance and reactance parts, respectively, of the impedance,

with

$$|Z| = \sqrt{R^2 + \left(\frac{1}{\omega C}\right)^2} \quad (11.19)$$

and

$$\Delta\varphi = \arctan\left(\frac{1}{\omega CR}\right). \quad (11.20)$$

11.4 Applications

There are many and very diverse applications of dielectric relaxation/impedance spectroscopy. The option of distinguishing between free and bound water makes these methods attractive to investigate corrosion [18, 133], changes in hydration in nanoporous systems [1] or transitions of thermo-responsive polymers [63, 118]. Dipole responses in biomaterials include studies covering a broad range of applications [62, 89, 119] from molecules [11, 34, 59, 130] via cells [84, 101, 103, 132] to tissues [27, 104]. In the framework of this book, two examples for application from the biosciences are being presented, both acquired in AC fields. The first one exemplifies the use of dielectric spectroscopy to identify and investigate specific dipole systems. The second one involves a miniaturized system developed for the purpose of biosensing.

11.4.1 Application to Colloidal Model Systems – Lipid Vesicles

Lipid membranes are frequently used as a biomimetic model scheme to investigate, in a controlled reduced system, basic properties and processes of biomaterial. In Fig. 11.7, a complete dielectric relaxation spectrum from a system of fully hydrated membranes of DMPC at ~ 308 K is shown, acquired with a coaxial probe that is connected to a network analyser [58]. Detailed analysis [13, 38, 58] exhibited two independently responding dipole systems that could be attributed to the polar headgroups of the lipids including their first tightly bound hydration shell of water and, as a second system, to a sequence of hydration layers. The headgroup system is relaxing with a pure Debye characteristic, signified by a relaxation frequency $f_1 = 41$ MHz, whereas the hydration shell behaviour is best described by an exponentially decaying distribution of relaxation times, centred around 345 MHz and well below the value of 19 GHz for bulk water.

From the temperature course as depicted in an Arrhenius diagram (see Fig. 11.8), typical activation energies were determined for the two relaxation processes, namely 42 kJ/mol for the phosphatidylcholine headgroups connected to the myristoyl chains of DMPC, and 32 kJ/mol for the relaxation of the bound water. Most remarkably, a small jump in the relaxation frequency of the headgroups at ~ 297 K indicates the gel/liquid main phase transition of DMPC. This results in a sudden reduction of chain packing with a related release of headgroup dipole mobility.

Such an appearance is absent in a system like DOPC ($T_m < 273$ K). Moreover, the presence of the kink in the oleoyl chain results in a lower chain packing and a related increase in the freedom of rotation for the attached choline group, which is evident in a reduced activation energy of 34 kJ/mol. Of course, the headgroup relaxation frequency is unchanged and likewise the hydration water along a DOPC interface exhibits essentially the same activation energy (31 kJ/mol) as measured for a DMPC interface.

Evidently, the dielectric spectrum allows tracing changes in molecular packing within the lipid membrane. Thus, it is a promising tool to monitor adhesions and insertions of, for example, drug molecules. One of the most obvious applications

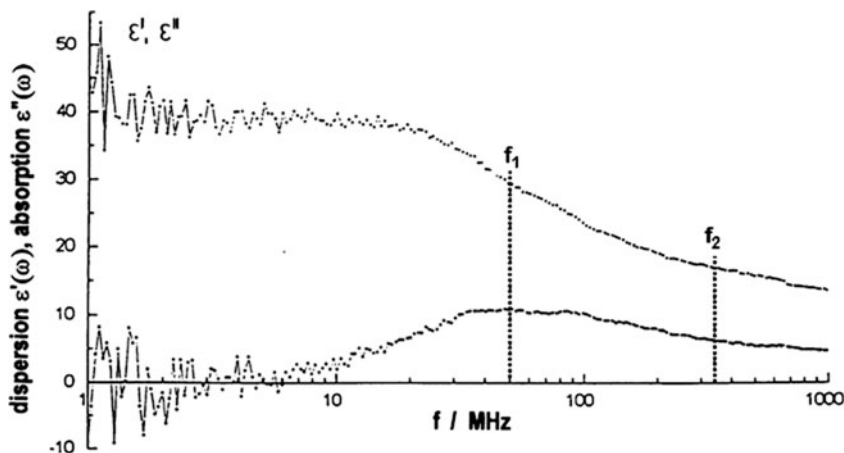
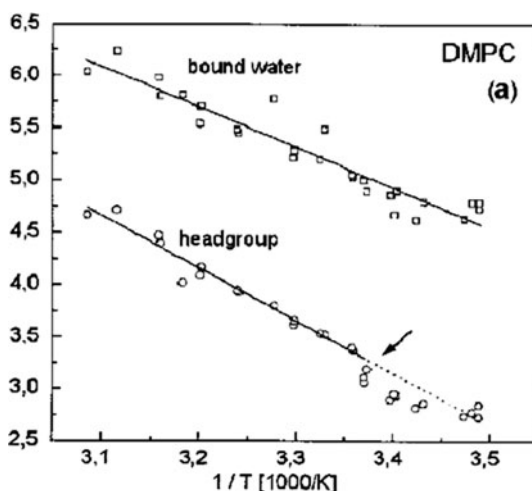


Fig. 11.7 Frequency-domain dielectric spectrum acquired on a system of lipid vesicles (DMPC). Analysis revealed two overlapping dipole resonances signified by the frequencies f_1 and f_2 .

Fig. 11.8 Separate Arrhenius plots of the relaxation frequencies from the two dipole systems of lipid headgroups (phosphatidylcholine) and interfacial water (hydration shells). Note the mobility jump upon phase transition at $\sim 23^\circ\text{C}$.



is the investigation of the state of interfacial water. Here, the dielectric spectra give insight into modifications in hydration [63, 76, 100, 119], for example due to solutes that change the local water structure (as sugars do) or upon surface binding of macromolecules that will disturb the hydration shell.

11.4.2 Application to Living Biomaterial

11.4.2.1 Lab-on-a-Chip Systems in General

Impedance measurements can be used to monitor cell behaviour in vitro [101] and in vivo [4, 45, 131]. Recently developed chip-based methods allow for spatial and

temporal control of cell growth and cell stimuli. These approaches lead to new microsystems, which are multifunctional platforms and permit the monitoring of basic biological parameters. Besides that, they may as well serve as cell-based sensors with implemented biochemical, biomedical, biophysical and environmental functions. These microsystems may imply many steps from the preparation of a cell culture sample, subsequent specific treatment and cell selection and, finally, biochemical analysis [105]. Such cell-based biosensors can report the physiological changes of the cells during the culturing and during treatment including their proliferation activity and changes in morphology. The chip read-out can be done using optical methods, for instance via fluorescence markers or microspectroscopy, and via an electrical output by measuring changes in the impedance or electrical potential as mentioned above, or via a combination of both methods [36].

Lab-on-a-chip devices are used in a wide variety of fields. They are being continuously developed, taking up the new trends and methods from technological developments. With modern techniques such as AFM and advanced fluorescence methods (e.g. single-particle tracking [31]), it is now possible to study single molecules in conditions that resemble *in vivo* circumstances. Another famous example is the Patch-Clamp technology, developed by Sakmann and Neher that allows studying single-molecule ion channels in living cell membranes [104]. All these methods are in detail very tedious and therefore mainly used in the experimental research field. Still, they are the foundation of chip-based methods which in the future shall serve as manageable tools in the application lab. Complex devices are being engineered that process multiples of events and reactions in order to simultaneously acquire manifold information, not restricted to only one type of molecule or an isolated reaction.

At the same time, sample volumes are minimized and observation scales shall reach sizes for features below the micrometre range. Therefore, lithographic approaches for processing hard and soft materials are used and combined with microfluidics and biochemical patterning [24]. An example for this continuing integration and minimization process is the development of planar parallel Patch-Clamp devices that make it possible to run 48 Patch-Clamp recordings at a time [14, 36]. The pre-patterning of active biomaterial on surfaces, known from AFM and from studies with optical tweezers [49], is now also used to specifically deposit and orient cells with respect to single chip elements [24].

Another application of lab-on-a-chip systems is the scaling down of chemical and biochemical analytical reactions. This is very attractive since smaller volumes of reagents are needed. This is more cost effective, the product output is faster and the production process is more environmentally appealing [55]. Miniaturization already started in 1999 when Agilent launched their 2100 Electrophoresis Bioanalyzer. This system contains a microfluidic-based platform for the analysis of DNA, RNA, proteins and cells. In 30 min 16 samples of reverse transcriptase–polymerase chain reactions (RT-PCRs) could be processed, an analysis that takes many hours with traditional methods. In continuation of the technological evolution, digital array chips were developed that carry microfabricated compartments for 9000 PCRs at a time [72]. This is only possible by use of semiconductor fabrication techniques from interdisciplinary crossover and exchange of technologies. Nowadays, solid-phase

and combinatorial chemistry and molecular biology micro-arrays are created with millions of probes for DNA and RNA diagnostics [55]. As another step of integration, medical diagnostics made use of both systems, PCR and DNA micro-arrays, and combined them for the detection of viral and bacterial pathogens [21, 105]. However, this kind of medical diagnostics is not restricted to DNA and RNA detection. Other approaches are immunological techniques that can be combined with chip technologies to detect virus particles or protein variations caused by infections [21] in a fast and reliable way. The amount of potential applications seems unlimited.

In summary, lab-on-a-chip systems constitute a new generation of devices for miniaturization and integration of a complex set of functions. Combined with microfluidic systems, there is now the opportunity to develop new diagnostic systems for health care. Lab-on-a-chip systems have many advantages, such as being inexpensive, precise and reliable, and they can easily be adopted for the creation of portable point-of-care systems.

11.4.2.2 Impedance Spectroscopy with IDES in Lab-on-a-Chip Devices

As an example for an integrated lab-on-a-chip system, cell-based sensors using interdigitated electrode structures (IDES) for measurements of cell impedances with the goal of monitoring physiological changes are presented [56].

With the cell-chip technology two different subjects can be addressed. First, different influences on cells during their cultivation on chips, such as temperature, chemical compounds or nutrition factors, can be monitored; second, basic biological insights in the cell and tissue behaviours can be obtained. For the monitoring of cell proliferation and morphology, impedance measurements are a non-invasive tool which allows measurement of cell kinetics simultaneous to cultivation. Invasive procedures such as collecting cell samples or addition of chemicals, as commonly used in molecular testing approaches, are avoided [46].

The electric field of planar electrodes on a chip does not extend deeply into the volume of the working solution. Therefore, signal-to-noise ratio is best and analysis is easiest for samples that are confined closely to the electrodes or, optimally, tightly attached to the chip surface. In a non-chip conformation, parallel electrodes at short distance may serve to investigate cells suspended in the enclosed volume between the electrodes.

In Fig. 11.9 an example of an integrated chip for the monitoring of cell proliferation is shown. The chip contains sensor electrodes for impedance measurements and sensors for the measurement of the physiologically relevant parameters of pH, pO_2 and temperature. For a detailed description of the chip, please refer to [Chapter 10](#) of this issue.

There are different protocols for impedance measurements. For example, the attaching and spreading of cells on a chip surface easily requires a period of hours or even days. In such a case, it is sufficient to restrict the impedance measurements to one defined test frequency. This optimal frequency differs among different cell types and needs to be determined ahead of the experiments using cells attached onto the chip surface [101]. The test frequency is then determined from the maximal

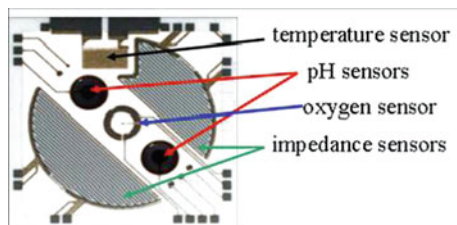
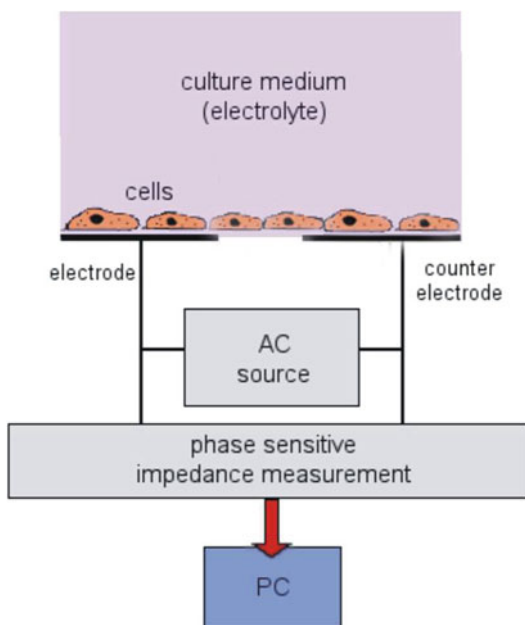


Fig. 11.9 Lab-on-a-chip device with integrated temperature, pH and oxygen sensors. For impedance measurements, IDES structures can be used (provided by cellasys GmbH, Munich, Germany).

Fig. 11.10 Principal set-up for the measurement of cell impedance of a confluent cell layer on a planar electrode structure



response obtained from full impedance spectra in the range of beta-dispersions (see Section 11.5).

There is non-linearity in the response of biological systems that requires the impedance measurements to be done at very low excitation amplitude with AC voltages in the order of ~ 10 mV_{pp} (pp: peak-to-peak). A general set-up for confluent cell layers is shown in Fig. 11.10.

A typical normalized impedance spectrum of attached cells and of cells after a Triton X-100 treatment is shown in Fig. 11.11. Healthy cells exhibit a characteristic course of the spectrum with a maximum at a specific frequency. Treatment of the cells with Triton X-100 leads to the destruction of the cell membranes and therefore to the detachment of the cells from the chip surface. As a consequence, the spectrum is modified: the signal amplitude around the initial maximum drops. Hence, the

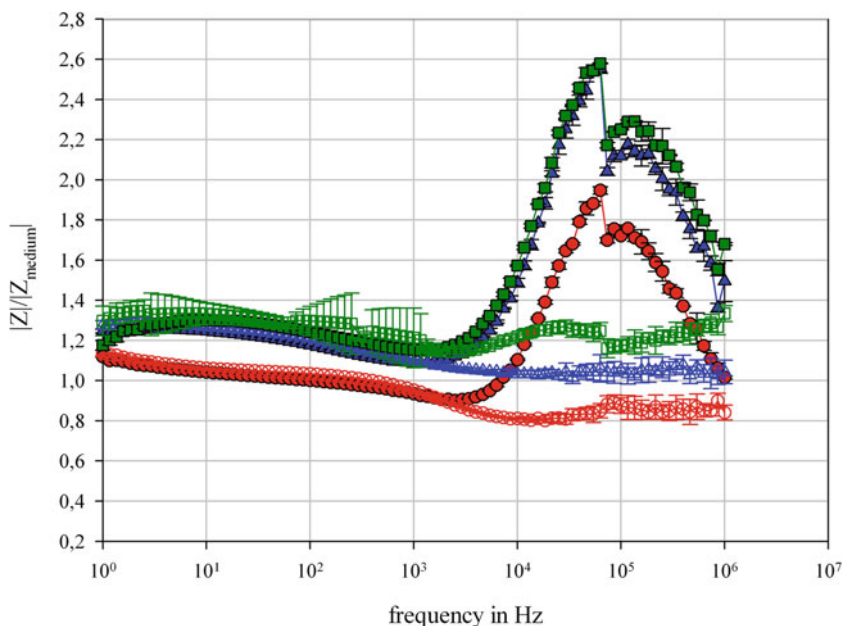


Fig. 11.11 Normalized impedance over frequency of three different chips with a confluent cell layer. Curves with full circles are impedance spectra of confluent cells before the treatment with 0.2% Triton X-100 (mean values). Curves with empty circle are impedance spectra of confluent cells after the treatment with 0.2% Triton X-100 (mean values). Data of the green, the blue and the red curves are obtained from the same chip, respectively. Error bars indicate the standard deviation.

system can be calibrated according to the change of signal amplitude at the specific test frequency to determine the mass of the adhered cells.

Further experiments can then be carried out using only this frequency, and the cell response towards different influences (e.g. chemical treatment and temperature) can be monitored over time. An example for an impedance measurement at this optimal test frequency is given in Fig. 11.12.

Figure 11.13 schematically shows the results from impedance measurements over time during the attachment of the cells onto the chip surface together with results from a measurement without any cells, both conducted at the same well-defined excitation frequency.

A model for the interpretation of such impedance measurements of cells on the surface of the chip, together with its electronic equivalent circuit diagram, is depicted in Fig. 11.14. The result from the experiments consists of a set of frequency-dependent complex impedances, given as imaginary and real parts of the impedance. These data can be entered into a Cole–Cole plot (see Fig. 11.15). Now, an attempt can be made to fit these data to a theoretical model. The physical parameters of the biological material under study can then be expressed as specific values in terms of resistances and capacitances that are entered into the fitting from

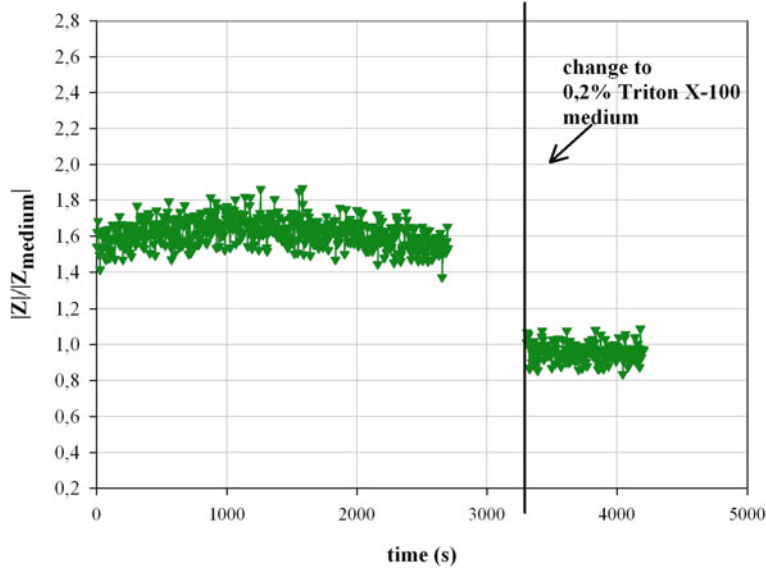


Fig. 11.12 Normalized values of the impedance of cells measured at their optimal test frequency over the time before and after the treatment with Triton X-100

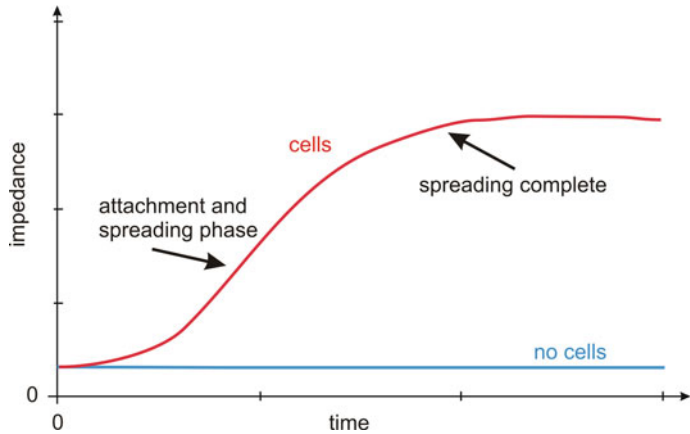


Fig. 11.13 Impedance measurement over the time during the attachment and spreading of cells on the chip surface (red curve) and without any cells (blue curve)

the model. Essentially, the parameters of an equivalent electrical circuit are fitted to the points of a measured impedance spectrum by means of complex least square fitting [8].

The basis of such a fit is derived from a properly chosen equivalent circuit like the one proposed in Fig. 11.14, right. It represents a simple model for the electrical current in a layer of biological cells on a chip. For the case given, it consists of

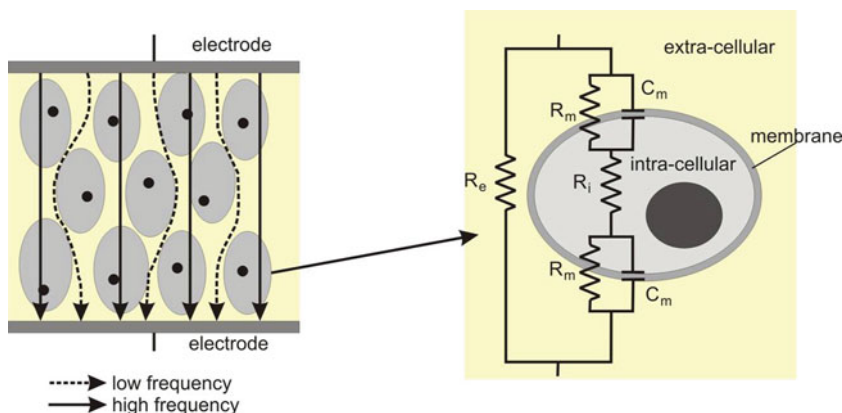


Fig. 11.14 Left: Current paths at high (full line) and low frequencies (dotted line) through a confluent cell culture on IDEs. Right: equivalent electrical circuit of a cell.

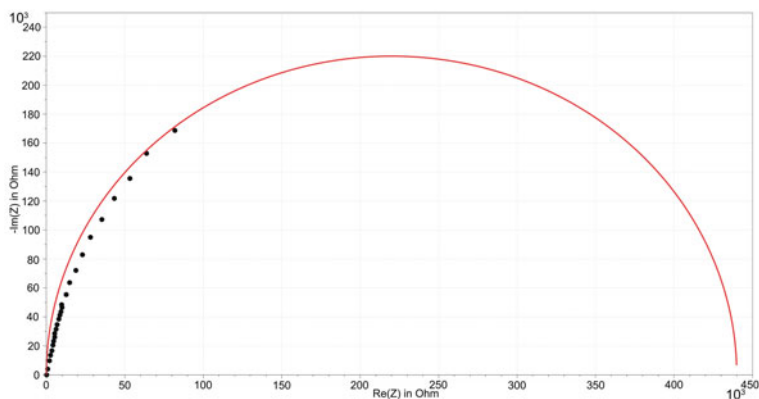


Fig. 11.15 Impedance spectra of a confluent cell layer (dots) and a fit on the equivalent circuit

two parallel current paths, one that runs exclusively through the extracellular liquid and a second current which flows through the cells. The extracellular liquid is represented by the resistance R_e , which is mainly dependent on the ionic composition of the fluid. In the case of tightly packed cells this current can be omitted. The second current is more complex and runs through the intracellular part of the cytosol represented by the resistance R_i and through a transmembrane part (two times, as the current crosses the cell membrane twice). In the model, this transmembrane part is entered as a parallel combination of a capacitor and a resistor. The capacitor is described by the capacitance of the cell membrane C_m , and the parallel resistor represents the membrane through which the leak current flows and is entered as a membrane resistance R_m [70]. On the left side in Fig. 11.14, a schematic top view

of the chip with the cultured cells is given. At low frequencies, the capacitor C_m acts like an open circuit and therefore most of the current flows through the pure Ohm part of the circuit, represented by R_e and R_m , R_i . R_m is in the range of $M\Omega$ and therefore exceeds typical values of R_e by far. Thus, the current flows mainly through the extracellular path (R_e) instead of through the cells. At high frequencies, the situation changes and the capacitor C_m acts like a short circuit. The alternating electric potential is transmitted via C_m into the intracellular volume and results in an intracellular current that is limited by R_i , in parallel to the leakage current across R_m . R_m contributes only a small portion and can be neglected in most cases. The total current is thus essentially composed of a transmembrane intracellular portion (C_m , R_i) and a parallel extracellular portion (R_e). The relative contributions depend on the packing density of the cells and on the composition of the intra- and extracellular ionic solutions.

The complex impedance for a resistor in parallel to a capacitor is given by

$$Z_{R||C} = \frac{R_m}{1 + i\omega R_m C_m} \quad (11.21)$$

Hence, the total complex impedance of the equivalent circuit shown in Fig. 11.14 is given by

$$Z_t = \frac{(2 \cdot Z_{R||C} + R_i)R_e}{R_e + R_i + 2 \cdot Z_{R||C}} \quad (11.22)$$

Complex Numbers

Complex numbers consist of two contributions, called the real and the imaginary parts. A complex number z may be written as

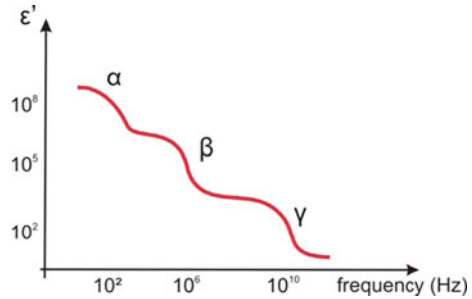
$z = x + iy$, where x signifies the real part and y the imaginary part of the number, and i is the imaginary unit with $i^2 = -1$. The numerical value (or modulus) of z is given as $|z| = \sqrt{x^2 + y^2}$. Complex numbers are often geometrically represented in Argand diagrams which resemble 2D vector diagrams, where z is then found as a position vector of length r under an angle (or phase) φ , resulting from the vector sum of x and y . Accordingly, z can be written in polar form as $z = r \cdot e^{i\varphi}$, and $|z| = r$.

The complex conjugate of z is z^* , defined as $z^* = x - iy$, such that $z \cdot z^* = |z|^2$, or $|z| = |z^*|$. This allows to extract the imaginary and real parts from z by use of the complex conjugate:

$$\operatorname{Re}(z) = \frac{1}{2} (z + z^*) \quad \text{and} \quad \operatorname{Im}(z) = \frac{1}{2i} (z - z^*).$$

Added by the editors

Fig. 11.16 Scheme of electric permittivity of biological materials (details see below). The α , β , γ drops with $\Delta\epsilon'_j$ with $j = \alpha, \beta, \gamma$ account for typical relaxation processes relevant for biomaterial (details see text).



The impedance Z can be represented in a Cole–Cole plot [20, 23, 51, 91] that gives a semicircle plot in the complex space. Figure 11.15 shows a complex non-linear fit of a measured impedance spectrum of a confluent cell layer to the equivalent electric circuit shown in Fig. 11.14. The impedance spectrum was measured in a finite frequency range up to 100 kHz, and the simulation was performed between 0 Hz and 100 MHz.

11.5 Phenomenological Relaxation Regions in Biomaterial

Electrical bioimpedance spectroscopy addresses the measurement of the electrical impedance of a biological sample. It can reflect some interesting physiological conditions and events such as changes in mobility and hydration, or it can serve as a phenomenological fingerprint of a tissue/cell type to be distinguished from another species. It thus supplements other system information, as proliferation rate, gene expression, oxygen consumption or local pH value. The passive electrical properties of materials can be described by their dielectric constants ϵ_i as well as by their electric conductivity σ . For biological tissue and for cells those parameters depend dramatically on the frequency. This frequency dependence is noted as dispersions (Fig. 11.16). The origin of dispersion in biological materials is briefly discussed in the following [41, 46, 106, 108].

At low frequencies (<1 MHz) the conductivity of the tissue is determined by the conductivity of the electrolyte in the extracellular space. The total conductivity depends dramatically on the volume of the extracellular space. Some materials show dispersion (α -dispersion) with a middle frequency in the kHz range. It is assumed that interactions between counter-ions (ions bound at slightly charged surfaces) and biological membranes are responsible for this type of dispersion. Furthermore, it is believed that polarization of structures in the membranes (headgroup regions) is also involved in α -dispersion [58]. At frequencies below α -dispersion, the relative dielectric constant increases dramatically up to 10^7 .

At radio frequencies (3 kHz–1 GHz), biological materials show the so-called β -dispersion with middle frequencies in the range of 100 kHz–10 MHz. The origin of this dispersion is the charging of intra- and extracellular ions and their interactions. At frequencies above β -dispersion, the impedance of the cell membranes can be neglected. An applied voltage causes a current which flows through the extracellular space as well as through the intracellular space/medium (Fig. 11.12, left). The β -dispersion can be superimposed by side dispersions caused by the relaxation of amino acids or by the charging of intracellular organelles.

At microwave frequencies (>1 GHz), biological materials show γ -dispersion which is mainly determined by the relaxation of water molecules in the material. Here, the presence of hydration shells can be detected, e.g. along lipid membranes [58]. The middle frequency is at 19 GHz, which is the relaxation frequency of free water. Frequency shifts can be caused by e.g. water-bounded proteins. Hydrated proteins show a large spectrum in the range from a few MHz to GHz that reflects the charged macro(ionic) molecule with its shells of bound water and electrolytes.

11.6 Conclusion

Dielectric relaxation spectroscopy (DRS)/electrochemical impedance spectroscopy (EIS) has turned out to be a powerful technique for studying dynamic properties in systems with polar components or reaction mechanisms in ionic solutions or at charged interfaces (electrodes, surfaces of cells, etc.). The beginnings of EIS can be traced to the work of Heaviside and Warburg, more than a century ago. Applications are as diverse as the investigation of corrosion processes (when the results of Epelboin and co-workers [18] in the 1960s propelled EIS into the forefront as a corrosion mechanism analytic tool [1, 74, 98, 133]), the properties of dissolved polymers [54, 62, 63, 78, 88, 118, 134, 136] and colloidal systems [9, 12, 17, 19, 29, 39, 53, 112, 115, 128]. The latter two topics directly connect to the biosciences as suspensions of isolated cells represent a special case of colloidal suspensions: accordingly, DRS and EIS are emanating techniques nowadays that are applied to colloidal systems including dissolved polymers [50, 51, 56, 62, 63, 87, 88, 89, 90, 135, 137] and cell suspensions [3, 20, 30, 39, 42, 43, 69, 75, 80, 86, 90, 94, 95, 103, 114], and even whole tissue [27, 79, 108, 137, 139]. A special and almost omnipresent case is the contribution of water that is found in hydration shells of dissolved ions [97, 100], polymers [59, 121, 126] and suspended colloids as cells [38, 40, 58, 59, 63, 76, 77, 80, 100, 121, 123, 129], behaving very different from the dielectric behaviour of bulk water [22, 77, 113]. Complex systems as an assembly of cells in a matrix of polymers and in aqueous electrolyte solution do however not obey the simple Debye law but require modifications that account for the geometry and related physical details of such distributed impedance systems [13, 17, 38, 52, 58, 66, 95, 96]. New technical developments as to the spectrometers [33], miniaturization of samples and probes [32, 61] and better mathematical modelling now

Abbreviations	Quantity	Unit, value
C_m	Membrane capacity	[F]; 1 F = 1 CV ⁻¹
e_0	Elementary charge	1.602 × 10 ⁻¹⁹ C; C: Coulomb
ϵ_0	Vacuum electrical permittivity	8.854 × 10 ⁻¹² · C ² J ⁻¹ m ⁻¹
ϵ'	Electric permittivity: real part of dielectric constant	
ϵ''	Imaginary part of dielectric constant	
f	Frequency; $\omega=2\pi f$	[Hz], Hertz
G	Conductance	[Ω^{-1}]; $G = 1/R$
i	Electric current	
I	Ionic strength	[mol/l]
ι	Imaginary unit	
k	Boltzmann constant	1.380×10 ⁻²³ JK ⁻¹
λ_D	Debye length	[m]
$\mu, \underline{\mu}$	Dipole moment	[D=Cm]; Debye
N_A	Avogadro constant	6.022 × 10 ²³ mol ⁻¹
Φ	Electrical potential	[V=JC ⁻¹]
$\Delta \Phi, (U)$	Electrical potential difference, (voltage)	[V], Volt
φ	Phase (of a wave)	[°]
ω	Angular frequency	[Hz]
P	Electric polarization	[Cm]
R	Resistance	[Ω], Ohm
R_m	Membrane resistance	[Ω]
σ	Conductivity	[Ω^{-1} m ⁻¹]
T	Thermodynamic temperature [122]	[K], Kelvin
T_m	Melting temperature	[K], Kelvin
z	Ionic charge number	

AFM	Atomic force microscopy
DMPC	Di-myristoyl-phosphatidylcholine
DOPC	Di-oleoyl- phosphatidylcholine
DNA	Deoxyribonucleic acid
IDES	Interdigitated electrode structures
RT-PCR	Reverse transcriptase–polymerase chain reactions
PCR	Polymerase chain reaction
RNA	Ribonucleic acid

allow applying DRS and EIS to many systems and in advanced applications such as continuous process monitoring [32, 56, 57, 67, 119, 127, 134, 136] and medical diagnosis [27, 32, 35, 37, 71, 79, 84, 139] including non-invasive monitoring of glucose concentrations for diabetes patients [15, 16, 82, 125, 129, 130].

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Part III

Genetics and Proteomics

Chapter 12

DNA Variations, Impaired Insulin Secretion and Type 2 Diabetes

Valeriya Lyssenko and Leif Groop

Abstract The success in dissecting the genetics of complex polygenic diseases like type 2 diabetes (T2D) until now has not been a trivial task. The picture has dramatically changed during the past years with the introduction of genome-wide association studies (GWAS). Today we know about 30 genetic variants increasing risk of T2D or influencing glucose/insulin levels. Most of them seem to influence the capacity of β -cells to increase insulin secretion to meet demands imposed by increase in body weight and insulin resistance. This probably only represents the tip of the iceberg, and refined tools will over the next few years provide a more complete picture of the genetic complexity of T2D. This will include not only the current dissection of common variants increasing susceptibility for the disease but also rare variants with stronger effects. For the first time we can with some confidence anticipate that the genetics of a complex disease like T2D really can be dissected.

Keywords Genetics · Complex disease · Polygenic · Linkage study · Genome-wide scan · Association study · Single nucleotide polymorphism · Insulin secretion · β -Cell function

12.1 Introduction

12.1.1 Evidence That Type 2 Diabetes Is Inherited

There is ample evidence that type 2 diabetes (T2D) has a strong genetic component. The risk of developing T2D is approximately threefold increased in first-degree relatives of a patient with T2D compared to subjects without family history of diabetes; this value is often referred to as the sibling-relative risk, λ_s value around 3.5 [1]. If one parent has diabetes the risk that offspring will develop the disease is about 40%,

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and if both parents have diabetes the risk is approximately 70% [2]. Intriguingly, the risk in the offspring seems to be greater if the mother rather than the father has T2D [3]. Very high concordance rates of T2D have been reported in monozygotic twins of 70% compared with 20–30% in dizygotic twins [4, 5]. Thus, approximately 70% of the variability of diabetes may be heritable.

It is clear that the change in the environment towards a more affluent Western life style plays a key role in the epidemic increase in the prevalence of T2D worldwide. This change has occurred during the last 50 years, during which period our genes have not changed. This does not exclude an important role for genes in the rapid increase in T2D, since genes or variation in them explains how we respond to changes in the environment and the environment always imposes a selective pressure on genes. An example of such selective pressure is the mutation of the Lac gene causing lactase persistence, i.e. ability to drink and absorb cow milk as adults with the domestication of cattle [6]. The *hypothesis* that *thrifty genes* could be a plausible explanation for the interaction between genes and environment was first introduced by Neel in 1962 [7]. During a long time of evolution humans have been subjected to long periods of famine and unpredictable food supplies. Neel proposed that individuals living in an environment with unstable food supply (as for hunters and nomads) would maximize their probability of survival if they could maximize storage of energy. Genetic selection would thus favour energy-conserving genotypes in such environments.

An alternative explanation has been proposed by which these changes can be the consequence of intrauterine programming, the so-called *thrifty phenotype hypothesis* [8]. The reason for this could be that poor intrauterine nutrition permanently programmes the body to a constant starvation state, which would lead to a low birth weight and increased risk of T2D later in life. However, a low birth weight could also be a consequence of impaired β -cell function. Children with a glucokinase defect have a decrease in insulin secretion and a low birth weight if they inherit mutation from the father [9]. Carriers of glucokinase mutations can secrete insulin, but their β -cells have a higher glucose threshold for glucose-stimulated insulin secretion. This was particularly apparent in children of diabetic mothers, since these children would be expected to have a high birth weight as a consequence of high glucose concentrations passing the placenta and thereby stimulating the fetal pancreas to produce increasing amounts of anabolic insulin.

12.1.2 Risk Factors Predicting Future T2D

Although risk factors for T2D seem to differ between different ethnic populations, a *family history of diabetes* consistently confers an increased risk of future T2D [1]; however, its relative effect decreases with increasing frequency of T2D in the population. Its predictive value is also relatively poor in young subjects whose parents have not yet developed the disease. Abdominal obesity and presence of the metabolic syndrome and low level of physical activity confer an increased risk of T2D [10, 11]. However, an *impaired insulin secretion*, especially when adjusted for

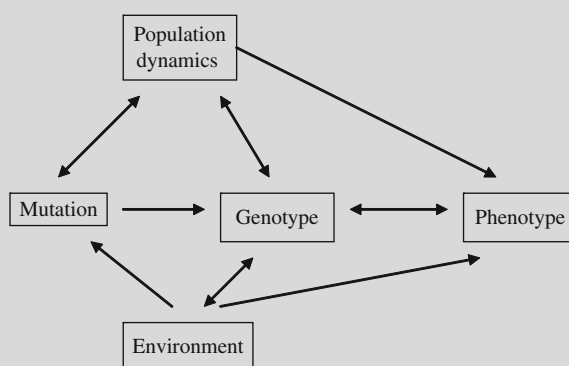
Genetic Epidemiology

Genetic epidemiology is the study of the role of genetic factors in determining health and disease in families and in populations and the interplay of such genetic factors with environmental factors.

It is closely allied to both molecular epidemiology and statistical genetics, but these overlapping fields each have distinct emphases. Traditionally, the study of the role of genetics in disease progresses through the following study designs, each answering a slightly different question:

- Familial aggregation studies: Is there a genetic component to the disease, and what are the relative contributions of genes and environment?
- Segregation studies: What is the pattern of inheritance of the disease (e.g. dominant or recessive)?
- Linkage studies: On which part of which chromosome is the disease gene located?
- Association studies: Which allele of which gene is associated with the disease?

Genetic epidemiology has developed rapidly in the first decade of the twenty-first century following completion of the Human Genome Project, as advances in genotyping technology and associated reductions in cost have made it feasible to conduct large-scale genome-wide association studies that genotype many thousands of single nucleotide polymorphisms in thousands of individuals. These have led to the discovery of many genetic polymorphisms that influence the risk of developing many common diseases.



The *classical* scope of genetic epidemiology

Further Reading:

Khoury MJ, Beaty TH, Cohen BH (eds) (1993) *Fundamentals of genetic epidemiology*. Oxford University Press, Oxford

Added by the editors

the degree of insulin resistance, the so-called disposition index, is the strongest predictor of future T2D. We have demonstrated this using two large prospective studies, the Malmö Preventive Project (MPP) and the Botnia Prospective Study (BPS) over a more than 25-year follow-up period. In both studies, obesity and a low insulin secretion when adjusted for insulin resistance were strongly associated with increased risk of future T2D, and this risk was doubled in individuals with a family history of diabetes (Fig. 12.1) [12]. For whether a family history of diabetes can be replaced by genetic testing in the prediction of T2D, see below.

12.1.3 Genetic Variability

Genetic mapping of an inherited disease implies the identification of the genetic variability contributing to the disease. Such variability can be deletions, insertions or changes in a single nucleotide in the genome, single nucleotide polymorphism (SNP). If an SNP results in a change in the amino acid sequence it is called a non-synonymous SNP. There are about 10 million SNPs in the human 3 billion bp genome, which means 1 SNP at about 300 bp intervals. SNPs in coding sequences (exons) are seen at 1250 bp intervals. Microsatellites are short tandem repeats of nucleotide sequences (e.g. CA) found at about 5000 bp intervals. Whereas SNPs are frequently bi-allelic, microsatellites have multiple alleles and are thus much more polymorphic than SNPs. Several public databases provide information on SNPs in different genes (e.g. www.ncbi.nlm.nih.gov/SNP). An SNP in a database is often

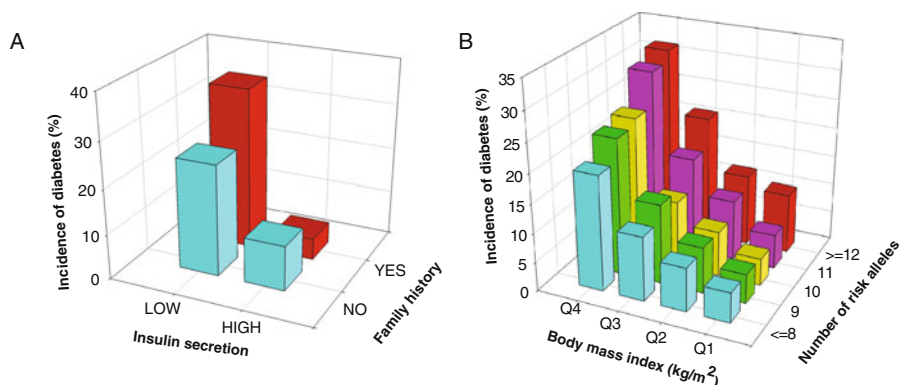


Fig. 12.1 Risk of incident T2D in individuals with different risk factors. (a) Family history of T2D doubled the risk of T2D associated with a low insulin response to oral glucose. (b) There was an increased risk of T2D with increasing quartiles of BMI and increasing number of the risk alleles. The risk was highest in subjects with high genetic risk and highest quartile of BMI yielding an OR of 8.0 as compared to those with low genetic risk and lowest quartile of BMI. The Y-axis shows incidence of diabetes.

referred to as dbSNP; at the moment (build 123) about 60% of all SNPs are in public databases.

An SNP can be either the cause of the disease (causative SNP) or a marker of the disease. This occurs when the disease susceptibility allele and the marker allele are so close to each other that they are inherited together, a situation called *linkage disequilibrium* (*LD* or *allelic association*). Such a combination of tightly linked alleles on a discrete chromosome is called a haplotype. While this region is characterized by little or no recombination (haplotype block), regions with high recombination rate usually separate haplotype blocks. LD thus describes the non-random correlation between alleles at a pair of SNPs; it is usually defined by D' or r^2 values. A D' value of 1 indicates that the two alleles are in complete LD, whereas values below 0.5 indicate low LD and a high recombination rate. LD extends over longer distances in isolated populations, but is also higher in European compared with African populations. This is considered to reflect a population bottleneck at the time when humans first left Africa.

An international joint effort to create a genome-wide map of LD and haplotype blocks is called the HapMap project (<http://www.hapmap.org/groups.html>). The hope is that by knowing the haplotype block structure of the genome, one could capture the genetic variability of the genome by genotyping a much smaller number of SNPs that describe the haplotype block (haplotype tag or htg SNPs).

Genotyping Arrays

In molecular biology and bioinformatics, an *SNP array* is a type of DNA microarray which is used to detect polymorphisms within a population. A single nucleotide polymorphism (SNP), a variation at a single site in DNA, is the most frequent type of variation in the genome. More than 10 million SNPs have been identified in the human genome. The map of SNPs serves as an excellent genotypic marker for research.

The three mandatory components of the SNP arrays are

- The array that contains immobilized nucleic acid sequences or target,
- One or more labelled allele-specific oligonucleotide (ASO) probes,
- A detection system that records and interprets the hybridization signal.

To achieve relative concentration independence and minimal cross-hybridization, raw sequences and SNPs of multiple databases are scanned to design the probes. Each SNP on the array is interrogated with different probes. Depending on the purpose of experiments, the amount of SNPs present on an array is considered. Several companies provide standard genotyping arrays that currently allow genotyping of 1 million SNPs and also include typing of structural variants like copy number variations (CNVs).

Adapted from http://en.wikipedia.org/wiki/SNP_array

Added by the editors

12.1.4 Mapping Genetic Variability

Profiling genetic variation aims to correlate biological variation (phenotype) with variation in DNA sequences (genotype). The ultimate goal of mapping genetic variability is to identify the SNP causing a monogenic disease or the SNPs increasing susceptibility to a polygenic disease. The most straightforward approach would be to sequence the whole genome in affected and unaffected individuals but this is for practical reasons not yet possible. Many indirect methods have been developed to achieve goals such as linkage and association approaches.

12.1.5 Linkage

The traditional way of mapping a disease gene has been to search for linkage between a chromosomal region and a disease by genotyping a large number (about 400–500) of polymorphic markers (microsatellites) in affected family members. If the affected family members would share an allele more often than expected by non-random Mendelian inheritance, there is evidence of excess allele sharing. The most likely explanation for excess allele sharing is that a disease-causing gene is in close proximity to the genotyped marker. Ideally, such a genome-wide scan would be carried out in large pedigrees where mode of inheritance and penetrance would be known. Since these parameters are not known and parents are rarely available in a complex disease with late onset, most genome-wide scans are performed in affected siblings with no assumptions on mode of inheritance and penetrance (non-parametric linkage).

The LOD score defines the strength of linkage. This takes into account the recombination fraction (θ), which is the likelihood that a parent will produce a recombinant in an offspring. If the parental genotype is intact in the offspring, the recombination fraction is 0 (loci are linked); for completely unlinked loci it approaches 0.5. The probability test of linkage is called the *LOD score* (logarithm of odds). Two loci are considered linked when the probability of linkage as opposed to the probability against linkage is equal to or greater than the ratio of 1000/1. A LOD score of 3 corresponds to an odds ratio of 1000/1 ($P < 10^{-4}$). In a study of affected sib pairs, a non-parametric LOD score (NPL) is presented. Although this threshold was developed for linkage mapping of monogenic disorders with complete information of genotype and phenotype, the situation for mapping complex disorders is much more complex. Lander and Kruglyak [13] have proposed that the LOD threshold for *significant genome-wide linkage* should be raised to 3.6 ($P < 2 \times 10^{-5}$) while that for *suggestive linkage* (would occur one time at random in a genome-wide scan) can be set at 2.2 ($P < 7 \times 10^{-4}$). In addition, they suggest to report all nominal P values < 0.5 without any claim for linkage. In reality each data set will have different thresholds based upon information on affection status, marker density, marker informativeness, etc. Therefore, these thresholds should be simulated using the existing data set before any claims of linkage can be made.

Accuracy of genotyping and exclusion of Mendel errors are important for the success but also the careful definition of affection status. This may not always be easy for diseases like asthma, schizophrenia or systemic lupus erythematosus (SLE). Even for diabetes the definition is based upon man-made cut-offs of plasma glucose. Dichotomizing variables may result in loss of power. One alternative is therefore to search linkage to a qualitative trait, e.g. blood glucose, blood pressure, body mass index (BMI) instead of diabetes, hypertension and obesity. Heritability (h^2) is often used as a measure of the genetic component of a quantitative trait. The higher the heritability, the more likely it is to find the genetic cause to the trait. Several statistical programs have been developed to support genome-wide scans of quantitative trait loci (QTL) like the variance component models SOLAR (www.sfbr.org/sfbr/public/software/solarR) and Merlin (www.sph.umich.edu/csg/abecasis/Merlin/tour).

Linkage will only identify relatively large chromosomal regions (often >20 cM) with more than 100 genes. Fine mapping with additional markers can narrow the region further but at the end the causative SNP or an SNP in LD with the causative SNP has to be identified by an association study. Several approaches have been described to estimate whether an observed association can account for linkage [14]. Without functional support it is not always possible to know whether linkage and association represent the genetic cause of the disease. This can, for many complex disorders, require a cumbersome sequence of in vitro and in vivo studies.

12.1.5.1 Candidate Genes from Linkage Studies

ADRA2A: The alpha2A adrenergic receptor (*ADRA2A*) has been known as a physiological mediator of adrenergic suppression of insulin release. It has previously been shown that *Adra2A* knockout mice present with enhanced insulin secretion and hypoglycaemia [15], and animals with β -cell-specific overexpression of *Adra2A* are glucose intolerant [16]. The diabetic Goto-Kakizaki (GK) rats display a major diabetes susceptibility locus on rat chromosome 1, called *Niddm1* [17]. A 16 Mb portion of *Niddm1*, termed *Niddm1i*, confers defective insulin secretion and is homologous to a region on human chromosome 10 that has been strongly associated with T2D [18, 19]. In a recent study, we have identified *ADRA2A* being overexpressed at the *Niddm1i* locus, and rats displayed elevated glucose levels which were paralleled by a profound reduction in insulin secretion [20]. Furthermore, we demonstrated that genetic variants in the *ADRA2A* gene in humans were associated with impaired early and second phase of insulin secretion, increased expression of both transcript and protein in human islets and increased risk of T2D (Fig. 12.2) [20]. This was the first time it was possible to merge genetic information from an animal model of T2D with genetic information in human T2D. Independently of these findings, a different variant in the *ADRA2A* gene has recently been associated with increased fasting glucose levels [21]. These findings could open up exciting possibilities for specific and tailor-made therapeutic intervention.

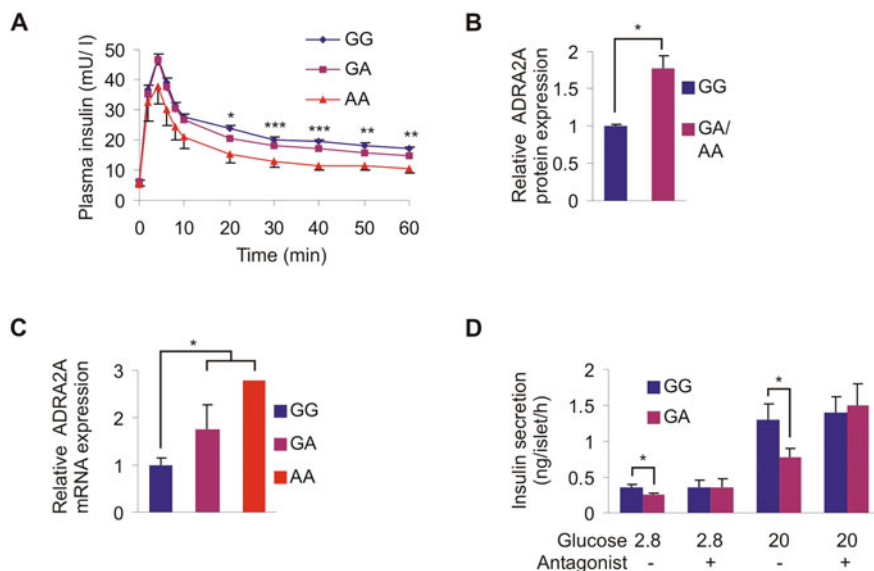


Fig. 12.2 Association of *ADRA2A* rs553668 with insulin secretion in humans. (a) Effects of rs553668 genotype on insulin levels during IVGTT in 799 individuals. Data are means $\pm$ SEM. (b) Immunoblots of total protein from human islets from eight individuals using polyclonal alpha(2A)AR antisera. The histogram shows average alpha(2A)AR signal normalized for β -actin from 4 blots from a total of 11 GG, 7 GA and 1 AA carriers. (c) Islet *ADRA2A* mRNA expression in 24 GG, 7 GA and 1 AA carriers. $P < 0.05$ for GG versus GA/AA, or $P < 0.05$ for linear regression of expression versus number of risk alleles. (d) Islet insulin secretion at 2.8 or 20 mM glucose with or without alpha(2A)AR antagonist. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

12.1.6 Association Studies – Candidate Genes

If there is a prior strong candidate gene for the disease, the best approach is to search for association between SNPs in the gene and the disease. This can be either a case–control or a nested cohort study. In a case–control study the inclusion criteria for the cases are predefined and thereafter matched individual controls are searched representing the same ethnic group as the cases. In a cohort study, affected and unaffected groups are matched, not individuals. Ideally cohorts are population-based but often they represent consecutive patients from an outpatient clinic. It is preferable that controls are older than cases to exclude the possibility that they still will develop the disease. If cases and controls are not drawn from the same ethnic group, a spurious association can be detected due to ethnic stratification.

One way to circumvent this problem is to perform a family-based association study. Distorted transmission of alleles from parents to affected offspring would indicate that the allele showing excess transmission is associated with the disease. The untransmitted alleles serve as control. This transmission disequilibrium test (TDT) represents the most unbiased association study approach but suffers from the

drawback of low power; only transmissions from heterozygous parents are informative. The prerequisite of DNA from parents usually enriches for individuals with an earlier onset of the disease.

PPARG: Even screening only one gene for SNPs can represent a huge and expensive undertaking. The peroxisome proliferator-activated receptor-gamma (*PPARG*) gene on the short arm of chromosome 3 spans 83,000 nucleotides with 231 SNPs in public databases; 7 of them are coding SNPs. The gene encodes for a nuclear receptor, which is predominantly expressed in adipose tissue where it regulates transcription of genes involved in adipogenesis. In the 5'-untranslated end of the gene is an extra exon B that contains an SNP changing a proline in position 12 of the protein to alanine. The rare Ala allele is seen in about 15% of Europeans and was in an initial study shown to be associated with increased transcriptional activity and increased insulin sensitivity and protective against T2D [22]. Subsequently, there were a number of studies, which could not replicate the initial finding. Using the TDT approach we could show excess transmission of the Pro allele to the affected offspring [23]. We thereafter performed a meta-analysis combining the results from all published studies showing a highly significant association with T2D. The Pro12Ala polymorphism of the *PPARG* gene is until now the best replicated gene for T2D ($P < 2 \times 10^{-10}$). It also predicts future T2D, especially in individuals with BMI > 30 kg/m² and fasting plasma glucose > 5.5 mmol/l and in carriers of risk variants in the *CAPN10* gene [24]. There is also a strong interaction with nutritional factors, and the protective effect of the Ala allele is enhanced with a high intake of unsaturated fat [25]. This may not be too surprising as free fatty acids have been proposed as natural ligands for *PPARG*.

KCNJ11: The ATP-sensitive potassium channel Kir 6.2 (*KCNJ11*) forms together with the sulphonylurea receptor SUR1 (*ABCC8*) an octamer protein that regulates transmembrane potential and thereby glucose-stimulated insulin secretion in pancreatic β -cells. Closure of the K-channel is a prerequisite for insulin secretion. A Glu23Lys polymorphism (E23K) in the *KCNJ11* gene has been associated with T2D and a modest impairment in insulin secretion [26, 27]. In addition, an activating mutation in the gene causes a severe form of neonatal diabetes [28]. Whereas these neonatal mutations result in a 10-fold activation of the ATP-dependent potassium channel, the E23K variant results in only a 2-fold increase in activity [29].

TCF7L2: By far the strongest association with T2D is seen for SNPs in the gene encoding for the transcription factor-7-like 2 (*TCF7L2*) [18, 30]. *TCF7L2* encodes for a transcription factor involved in Wnt signalling. Heterodimerization of *TCF7L2* with β -catenin induces transcription of a number of genes including intestinal proglucagon. It is clear that risk variants in *TCF7L2* are associated with impaired insulin secretion and an impaired incretin effect, i.e. impaired stimulatory effect of incretin hormones like GLP-1 and GIP on insulin secretion (Fig. 12.3) [31, 32]. It is also possible that the gene is involved in proliferation of β -cells in response to increased demands. At the onset of diabetes, T2D patients show a markedly increased expression of *TCF7L2* in their islets [31]. Since overexpression of *TCF7L2* in human islets resulted in impaired insulin secretion it is unlikely that

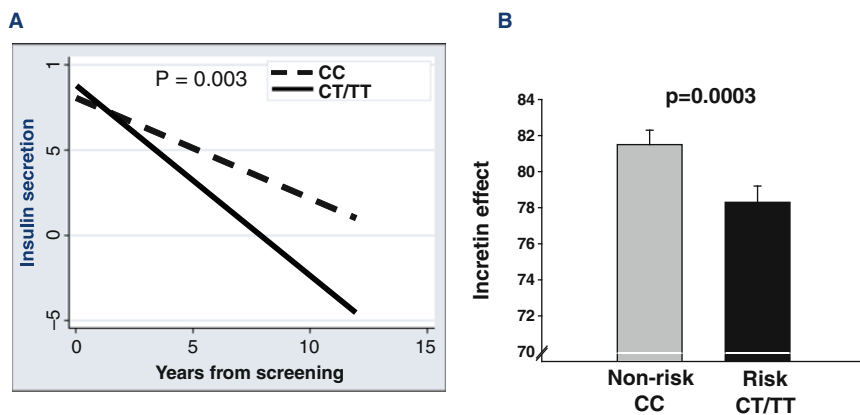


Fig. 12.3 Insulin secretion and incretin effect according to different *TCF7L2* rs7903146 genotypes. (a) Change in insulin secretion (disposition index) over time in subjects who converted to T2D in the Botnia cohort (risk genotypes = solid line). (b) Risk genotype carriers of the *TCF7L2* rs7903146 (risk genotypes = black bars) show impaired incretin effect as shown by lower insulin response to oral than to intravenous glucose.

the increased expression is a consequence of a defect in the downstream pathway; it rather reflects a defect in transcription or translation of *TCF7L2* itself. There is a unique splicing pattern of the *TCF7L2* gene in human islets, where especially exon 4 including isoforms are abundant [33]. Intriguingly, there was a positive correlation between the amount of exon 4 in human islets and HbA1c. Despite the increased expression of *TCF7L2* in islets it is not known whether risk variants in the gene cause increased or decreased Wnt signalling. On the other hand, in rodent islets disruption of *TCF7L2* also results in impaired insulin secretion [34]. Very recently, a new study has shown that the key SNP in the *TCF7L2* is located in a chromatin-free region and the risk allele increased the transcriptional activity of the gene [35]. It will be one of the greatest challenges to identify the underlying mechanisms, as *TCF7L2* undoubtedly represents a potential novel drug target in T2D.

WFS1: The Wolfram syndrome 1 (*WFS1*) gene encodes for wolframin, a protein, which is defective in individuals with the Wolfram syndrome. This syndrome is characterized by diabetes insipidus, juvenile diabetes, optic atrophy and deafness. *WFS1* gene was identified through candidate gene search of 1536 SNPs in 84 genes and further replication in 9533 cases and 11,389 controls [36]. Genetic variants in the *WFS1* have been associated with impaired glucose- and GLP1 (glucagon-like peptide-1)-stimulated insulin secretion [37–39]. *Wfs1* knockout mice showed progressive β -cell loss and impaired insulin secretion [40]. We have recently demonstrated that expression of *WFS1* gene is also perturbed in pancreatic islets and skeletal muscle [41]. However, the discovery of *WFS1* also highlights some of the difficulties of candidate gene studies. We are limited by our own imagination and only 1 out of 84 candidate genes gave a positive result!

Pattern Recognition in Gene Analysis and the Hidden Markov Model (HMM)

The sheer length of the human genome (3×10^9 base pairs) excludes analysis by expert eyes: the power of modern computers and a variety of algorithms have to be exploited. For genome-wide association studies (GWAS), there are many different tasks, algorithms and ready-for-use software systems. All that can be considered as *pattern recognition* problems. How is that?

While modern machinery delivers precise sequences of base pairs in a short time, relatively cheap and without ambiguities, the very problem is to read the meaning of the data. We may want, for example, to distinguish the 98.5% of non-coding (NC) regions from the 1.5% of genes (GE). In speech recognition, this task corresponds to the separation of noise from voice or to the task of associating a distinct phoneme out of a list of *hidden variables* to a received acoustic input, the *observable*.

So, in basic genome analysis, we may distinguish two different hidden states (NC versus GE) for a given single entry *A, C, G, T* in the sequence. In principle, this is easy to decide by machine when we know in advance (or gathered from test sequences and test algorithms) the following 12 probabilities: 4 probabilities for the possible four *transitions* between the two hidden states (here we assume that the sequence is a *Markov chain*, i.e. roughly speaking, a process without memory where the probability of one hidden state as next outcome solely depends on the preceding hidden state); and 4 outcome probabilities (for the outcomes *A, C, G, T*, respectively) for each of the two basic *stochastic variables* NC and GE. Then the *Viterbi algorithm*, a maximum a posteriori probability (MAP) argument, selects the most probable path of hidden variables for a given sequence of base pairs.

Further Reading:

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Added by the editors

12.1.7 Genome-Wide Association Studies

A problem in the field of genetics of complex diseases is the difficulty to replicate an initial association. The International HapMap Project (<http://www.hapmap.org>) and a public–private SNP consortium (<http://www.ncbi.nlm.nih.gov>) have provided a catalogue of 10 million common genetic variants in the human genome. With the improvement of genotyping technology it has become technically possible to genotype a large number of SNPs at affordable costs, which paved the way for the so-called genome-wide association studies (GWAS). In 2007 GWAS made a real breakthrough in the genetics of T2D applying DNA chips with >500,000 SNPs in a large number of patients with T2D and controls [42]. In our collaborative study with the Broad Institute and Novartis (Diabetes Genetic Initiative, DGI) we performed a GWAS in 1464 patients with T2D and 1467 non-diabetic control subjects from

Finland and Sweden. Prior to publication we shared the results with researchers from the FUSION (Finnish USA Study of NIDDM) and WTCCC (Wellcome Trust Case Control Consortium) groups [43]. We only considered positive results, which were seen and replicated in all three studies, i.e. together with replication samples the results were based upon DNA from 32,000 individuals. Two other GWAS [44, 45] in T2D have been published in the past year supporting and complementing our results.

Notably, *TCF7L2* was on top of each GWAS with a joint *P* value in the three scans of 10^{-50} [30]. Several of the new genes seem to influence β -cell proliferation by interfering with the cell cycle, e.g. *CDKAL1* and *CDKN2A/CDKN2B*. It has also become evident that some of the same SNPs which increase risk of or protect against T2D increase susceptibility to certain cancers like in the prostate and colon [46]. This has led to a hypothesis that genes increasing cell proliferation predispose to cancers but protect against T2D, and vice versa inability of β -cells to increase cell proliferation predispose to diabetes and, if occurring in other organs, protect against cancer [47]. In general, the risk associated with these variants was modest with odds ratios in the range of 1.2–1.5. This was only the beginning; an extended meta-analysis of GWAS data from more than 60,000 individuals (DIAGRAM) identified variants in or around 6 additional genes (*JAZF1*, *THADA*, *CDC23*, *LGR5*, *ADAMTS9*, *NOTCH2*) [30] with even more modest effect sizes than those seen for the initial variants. A third meta-analysis of even more individuals (>100,000) is soon to be published.

Most of the genetic variants described to date result in impaired β -cell function. When we compared high-risk and low-risk genotypes, i.e. those belonging to highest and lowest 20%, high-risk genotypes did not influence BMI or insulin sensitivity but they could not increase their β -cell function to compensate for the decrease in insulin sensitivity imposed by an increase in BMI [12] (Figs. 12.4 and 12.6). In addition, the GWAS have been extended to quantitative traits like glucose and insulin and have recently identified variants in about 15 loci to be consistently associated with glucose and/or insulin concentrations [21, 48–51].

KCNQ1: *KCNQ1* is an ATP-dependent potassium channel expressed in most tissues. Mutations in the gene cause the long QT-syndrome characterized by severe arrhythmias [52]. Two small (100 K chips) Japanese GWAS identified variants in this gene being associated with T2D [53, 54]. We have further replicated these findings in Scandinavian populations and also shown that variants predict future T2D, which partially can be explained by an effect on insulin secretion [55]. The question rose as to why it was missed in the previous European GWAS. The most likely explanation is that the risk genotype was seen in >90% of Europeans thereby limiting the power to detect an association.

MTNR1B: An intriguing observation was that a common variant in the gene encoding the melatonin receptor 1B (*MTNR1B*) was associated with impaired insulin secretion, elevated glucose concentrations and increased risk of future T2D (Fig. 12.5) [49]. There is a well-established link between sleep disorders and T2D. Preliminary data suggest that variants in the *MTNR1B* gene can partially explain this association. We could also show that the *MTNR1B* gene is expressed in human

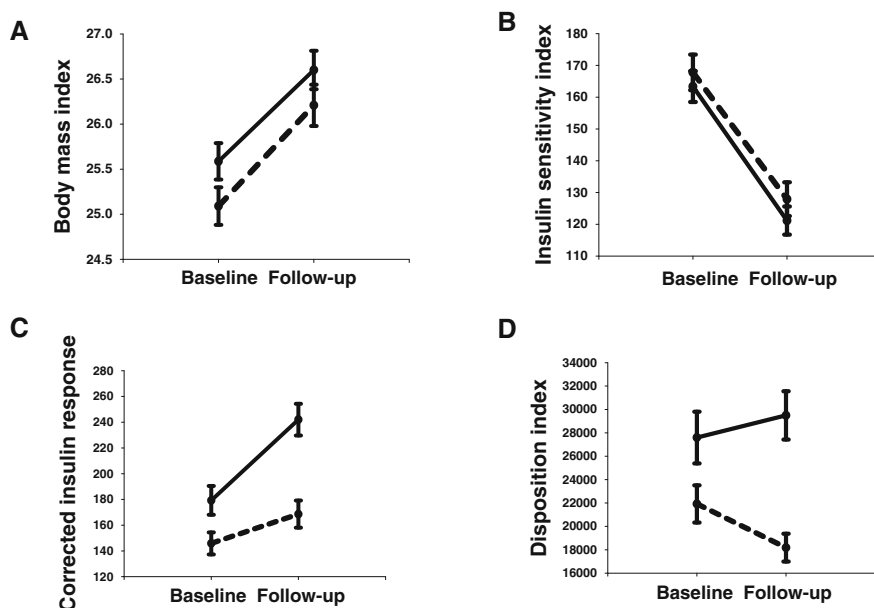


Fig. 12.4 Carriers of high-risk genotypes for T2D (solid line) cannot increase their insulin secretion to compensate for the increase in insulin resistance compared with carriers of low-risk genotypes (dashed line). High and low risk was defined as highest or lowest 20% of risk genotypes.

islets. However, we do not know whether melatonin is produced in islets from serotonin or transported to the islets. The *MTNR1B* gene was up-regulated in carriers of risk genotypes suggesting a gain-of-function effect increasing risk of T2D. In support of this, adding melatonin to clonal β -cells inhibited glucose-stimulated insulin secretion (Fig. 12.5). Inhibition of melatonin effects in islets could thus represent a novel therapeutic target.

12.1.8 Common Variants in MODY Genes

Maturity-onset diabetes of the young (MODY) is an autosomal dominant form of diabetes, where a mutation in a single gene causes the disease. There are at least six forms of MODY caused by mutations in a distinct gene. Most forms of MODY are caused by mutations in different transcription factors, i.e. *HNF4A* (MODY1), *HNF1A* (MODY3), *IPF-1* (MODY4), *HNF1B* (MODY5) and *NEUROD1* (MODY6), and only MODY2 is caused by mutations in the gene encoded for glucokinase enzyme (*GCK*) [56]. Common to all MODY carrier phenotypes is that they are characterized by impaired insulin secretion and usually show strong allelic variability, i.e. different mutations cause the disease in different families. We therefore looked at whether common variations in these genes

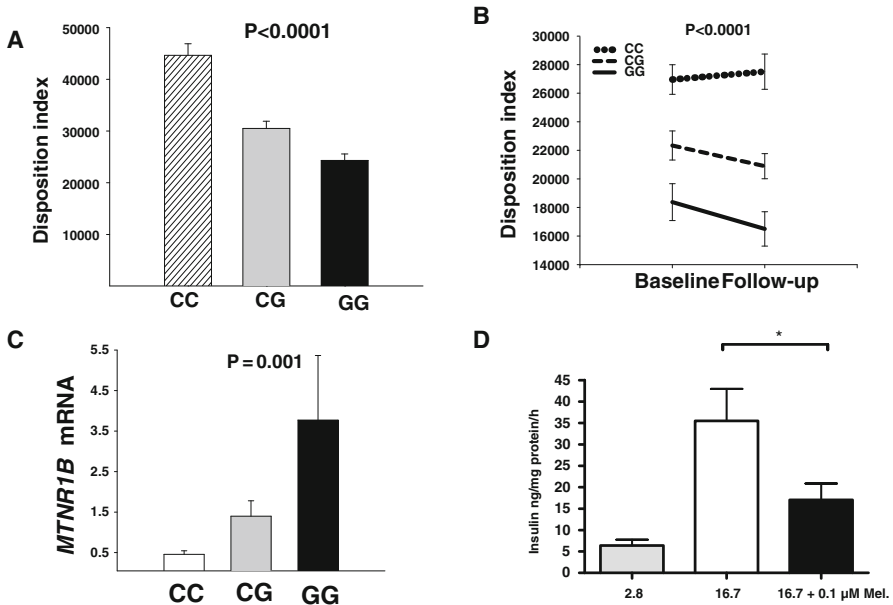


Fig. 12.5 Risk genotype carriers of the *MTRN1B* gene (black bars and solid lines) show (a) impaired β -cell function corrected for insulin sensitivity (disposition effect) cross-sectionally, (b) decline in β -cell function over 8-year follow-up period and (c) increased expression of the gene in human islets. (d) Melatonin inhibits glucose-stimulated insulin secretion.

could contribute to common late-onset T2D. This turned out to be the case, and we indeed demonstrated that common variants in *HNF1A*, *HNF1B* (*TCF2*) and *HNF4A* were associated with impaired β -cell function [57–59]. It was, however, not easy to detect these subtle effects, which seem to be unmasked in insulin-resistant obese elderly individuals. As mentioned above, the same variant in the *HNF1B* gene which increases risk of prostate cancer protects against T2D [46].

12.1.9 Personalized Prediction of T2D Risk?

Combined information of genetic and clinical risk factors ultimately might aid in personalized prediction of disease risk. Recently we have evaluated effect of clinical and genetic factors to predict progression to diabetes in two prospective cohorts [12]. When we replaced family history with increasing number of T2D-associated risk alleles, the risk of T2D gradually increased with increasing number of risk alleles and increasing quartiles of BMI. The risk was highest in subjects with high genetic risk and highest quartile of BMI yielding an OR of 8.0 as compared to those

with low genetic risk and lowest quartile of BMI (Fig. 12.1b). As these studies comprised a large number of participants with long follow-up we were in a unique position to address the question of whether genetic risk factors added to clinical risk factors could improve prediction of future diabetes. The results demonstrated that adding genetic markers to the clinical risk factors modestly improved the discriminatory power as assessed by the area under the receiver operating characteristic (ROC) curves (from AUC 0.73 to 0.74) [12]. An important factor defining the discriminative power of clinical and genetic risk factors is duration of follow-up. We also assessed the area under the ROC curves to determine the discriminative ability of clinical and genetic risk factors in relation to quintiles of follow-up time. We observed a decrease in AUC for the clinical model ($P = 0.01$) and an increase in the AUC for the genetic risk score ($P = 0.01$) with increasing duration of follow-up. These findings suggest that an individual genetic profile could be valuable from birth, long before exposure to most environmental risk factors takes place. It has been suggested that using a larger number of common variants can make possible an accurate prediction of genetic risk [60, 61]. Simulation studies have estimated that for an AUC of 0.80 to predict cardiovascular disease it will be necessary to genotype 50 risk variants with allele frequencies of 10% and ORs of 1.5 [62]. However, this figure might look different if we find rare variants with stronger effect size, e.g. with odds ratio $>2-3$, possibly requiring about 10 rare variants with strong effect size to explain the genetic risk of T2D. This figure might change completely if biomarkers involved in the key pathway could be identified, which together with genetic markers would markedly increase the risk prediction. Taken together, genetic tests cannot be offered yet to predict disease. The main reason is the marginally increased risk associated with each risk variant. However, it may be possible to use them on a population level to reduce the number of individuals needed to be included in trials aiming at prevention of T2D.

12.1.10 Pharmacogenetics

An important goal of genetics is to use the information to improve treatment, i.e. to identify individuals who are more likely than others to respond to a specific therapy.

While there are some intriguing examples on the potential of pharmacogenetics for monogenic forms of diabetes [63, 64], its role in the more common forms is still unclear. Patients with neonatal diabetes due to mutations in the *KCNJ11* gene do much better on treatment with sulphonylureas than with insulin [63]; in fact the sometimes accompanying developmental defects improve. Patients with mutations in glucokinase (*MODY2*) can be taken off all treatments including insulin as the disease does not progress and patients with mutations in *HNF1A* (*MODY 3*) respond extremely well to sulphonylureas [65]. It has also recently been shown that individuals with the risk genotype in *TCF7L2* respond poorly to treatment with

sulphonylureas, eventually as a consequence of their more severe impairment in β -cell function [66]. It is still an open issue whether variants in the *TCF7L2* would modify response to incretin mimetics.

There is some indication that variants in genes metabolizing/transporting metformin influence its effects [66, 67]. The effect one would like to see is that seen with the rs4149056 variant in the gene for *SLCO1B1* gene (a cation transporter) for prediction of myopathy during high-dose statin therapy; the variant was associated with a sixfold increased risk of myopathy [67].

12.2 Clinical Implications and Future Directions

Although it has been debated whether impaired insulin secretion or increased insulin resistance is the key defect in the pathogenesis of T2D, the accumulating evidence today points at the central role of the failing β -cells (Fig. 12.6). Dissecting the genetic architecture of a complex disease such as T2D is a rather challenging task. The genetic variants detected represent common variants shared by a large number of individuals but with modest effects. Each risk allele increases risk of T2D only by 12% [12]. The about 30 T2D genes discovered thus far explain only a small proportion (≈ 0.3) of the individual risk of T2D (λ_s of 3). It is still possible that there are rare variants with stronger effects not detected with current methods. It is

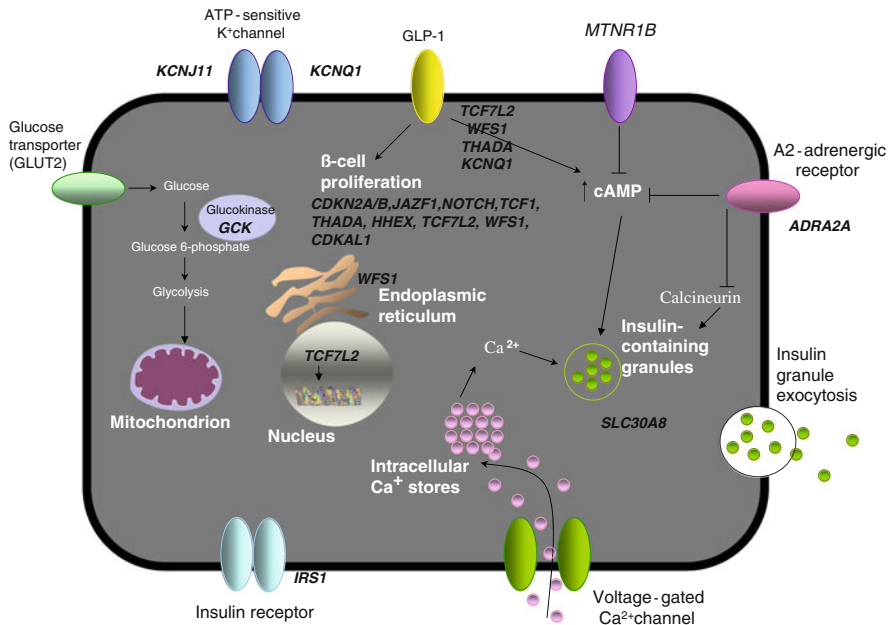


Fig. 12.6 Schematic view of a potential role of genetic loci influencing risk of T2D and/or glycaemic traits in β -cell function

unlikely that high-density DNA arrays can detect these rare variants. Their detection will rather require sequencing. Sequencing of the whole genome was once a dream, but with new technologies this dream may become true in a very near future. Dissection of the genetic complexity of T2D and identifying novel pathways in the pathogenesis of the disease most likely will pave ways to new therapeutic strategies.

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Chapter 13

Genetically Programmed Defects in β -Cell Function

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Abstract A significant level of insight into the machinery and workings of the pancreatic β -cell originates from the study of naturally occurring mutations in genes that encode the various components. Identifying these mutations has been important not only for tailoring treatment towards the specific subtype of diabetes associated, but also for highlighting the importance and pivotal role of a number of elements along the pathway of glucose-stimulated insulin secretion within the pancreatic β -cell.

Keywords β -cell · Mutation · K_{ATP} channel · Glucokinase · Maturity-onset diabetes of the young (MODY) · Hyperinsulinaemic hypoglycaemia (HH) · Neonatal Diabetes · Endoplasmic Reticulum stress

13.1 Introduction

The pancreatic β -cell and insulin secretion pathway are central to the pathophysiology of diabetes. Although the vast majority of diabetes is categorized as type 1 or type 2 diabetes, approximately 5% of cases have other specific causes including monogenic diabetes, i.e. diabetes resulting from the mutation of a single gene. Most of these naturally occurring mutations affect components of the β -cell with consequences severe enough to commonly cause development of diabetes in childhood or adolescence. Identifying the genes affected in monogenic β -cell dysfunction has lent considerable insight into the regulation of insulin secretion as well as guiding more accurate and relevant clinical management of patients. It is increasingly

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clear that defining these forms of β -cell dysfunction according to underlying genetically programmed defects is a more accurate and informative way of studying these disorders, rather than according to clinical phenotype which in these instances often overlap despite different genetic aetiologies. Box “Monogenic Forms of Diabetes” in [Chapter 13](#) gives a comprehensive summary of monogenic forms of diabetes.

Monogenic Forms of Diabetes (www.monogenicdiabetes.org and www.diabetesgenes.org)				
<i>Neonatal Diabetes Mellitus (NDM)</i>: Rare; incidence of at least 1 in every 260,000 live births and is diagnosed under the age of 6 months (Slingerland et al 2009)[119]. <i>Permanent Neonatal Diabetes Mellitus (PNDM)</i>: 50% of all cases of NDM. <i>Transient Neonatal Diabetes Mellitus (TNDM)</i>: 50% of all cases of NDM. <i>Maturity-onset Diabetes of the Young (MODY)</i>: 1 to 5% of all cases of diabetes.				
Type of diabetes	Gene or syndrome	Affected protein	Usual age of onset	References
PNDM and TNDM	<i>KCNJ11</i>	Kir6.2 (most common type of PNDM)	86% of cases diagnosed before 8 weeks. Median age of diagnosis 5 weeks TNDM cases have a median remission of 45 weeks	[30]
PNDM and TNDM	<i>ABCC8</i>	SUR1 – sulphonylurea receptor 1	86% of cases diagnosed before 8 weeks. Median age of diagnosis 4 weeks TNDM cases have a median remission of 22 weeks	[30]
PNDM	<i>INS</i>	Insulin (10–20% of PNDM)	Both autosomal dominant (AD) and autosomal recessive (AR) forms. The AR form presents earlier (median 1 week vs. 10 weeks for AD form)	[36]

Type of diabetes	Gene or syndrome	Affected protein	Usual age of onset	References
PNDM	<i>GCK</i>	Glucokinase	Typically diagnosed in first week	[92]
PNDM	<i>IPF1</i>	Insulin promoter factor 1	Typically diagnosed in first week	[115]
PNDM	<i>PTF1A</i>	Pancreas transcription factor 1 A	At birth	
PNDM	<i>FOXP3</i> , IPEX syndrome	Forkhead box P3	Typically diagnosed in first week	[110]
PNDM	<i>RFX6</i>	Transcription factor Rfx6	Autosomal recessive form of PNDM which presents in the first few days of life	[120]
PNDM	<i>EIF2AK3</i> , Wolcott–Rallison syndrome	Eukaryotic translation initiation factor 2-alpha kinase 3	Median age of diagnosis 10.5 weeks	[111]
TNDM	<i>Chr 6q24 (ZAC/HYMAI)</i>	<i>Zinc finger associated cell cycle (ZAC)</i> also known as pleomorphic adenoma gene-like 1 (<i>PLAG1</i>). <i>HYMAI</i> : hydatidiform mole-associated and imprinted transcript. Most common form of TNDM	Usually diagnosed in the first 4 weeks with median age of remission 14 (5–60) weeks	[30]
TNDM	<i>HNF1B</i>	Hepatocyte nuclear factor 1B (HNF1 beta)	Birth to 6 months	[22]
HNF4A-MODY	<i>HNF4A</i>	Hepatocyte nuclear factor 4 α (HNF4 alpha)	Adolescence or early adulthood	[26]
GCK-MODY	<i>GCK</i>	Glucokinase. GCK-MODY is the second-most common subtype of MODY	Mild hyperglycemia from birth. Often asymptomatic and only detected on routine blood glucose testing	[92]

Type of diabetes	Gene or syndrome	Affected protein	Usual age of onset	References
HNF1A-MODY	<i>HNF1A</i>	Hepatocyte nuclear factor 1 α (HNF1 alpha) HNF1A-MODY is the most common subtype of MODY	Adolescence or early adulthood	[26]
IPF1-MODY	<i>IPF1</i>	Insulin promoter factor 1	Very rare similar presentation to HNF1A-MODY	[81, 82]
HNF1B-MODY	<i>HNF1B</i>	Hepatocyte nuclear factor 1B (HNF1 beta)	Adolescence or early adulthood usually presents with renal abnormalities	[81, 82]
NEUROD1-MODY	<i>NeuroD1</i> or <i>BETA2</i>	Neurogenic differentiation factor 1	Very rare similar presentation to HNF1A-MODY	[81, 82]
CEL-MODY	<i>CEL</i>	Carboxy ester lipase (CEL)	Adolescence or early adulthood. May present with clinical features of pancreatic exocrine deficiency	[106]
INS-MODY	<i>INS</i>	Insulin	Adolescence or early adulthood	[80]
WFS	<i>WFS1</i>	Wolframin	Also known as DIDMOAD (<i>d</i> iabetes <i>i</i> nsipidus, <i>d</i> iabetes <i>m</i> ellitus, <i>o</i> ptic <i>a</i> trophy and <i>d</i> eafness) is a syndrome that includes diabetes, optic atrophy and deafness. Median age of presentation of diabetes is 6 years	[8]

Susceptibility genes for the more common forms of T2D are also providing novel insights into β -cell function, indeed emphasizing its key role in diabetes pathogenesis (over and above insulin resistance) and will be dealt with in detail

in Chapter 12. It is interesting to note the overlap between genes involved in monogenic forms of β -cell dysfunction and T2D. For a number of genes (e.g. *KCNJ11*, *GCK*) there are clear examples of an allelic spectrum where genetic variants influence glycaemic control with differing degrees of severity [38, 40, 41, 42, 51, 88, 128, 137].

This chapter will trace the pathway of glucose from its uptake through metabolism and stimulation of insulin secretion and via each β -cell component, and its affecting naturally occurring mutations demonstrate the key role that each play in the biology and system of the pancreatic β -cell.

13.2 The Pancreatic β -Cell, Insulin Secretion and the Main Targets of Genetically Programmed Defects

Figure 13.1 is a schematic representation of the pancreatic β -cell showing the main components involved in glucose-stimulated insulin secretion as well as the sites of the main mutations affecting β -cell function.

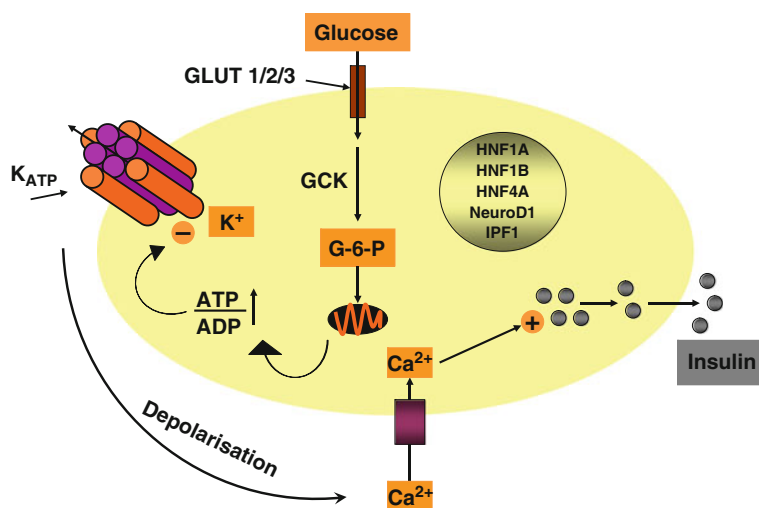


Fig. 13.1 The pancreatic β -cell: location of proteins mutated in humans which cause monogenic forms of β -cell dysfunction. Glucose enters the pancreatic β -cell via the glucose transporter. Inside the cell glucose is phosphorylated by glucokinase (GCK) to glucose-6-phosphate (G6P) in the first rate-limiting step of glucose metabolism. G6P is metabolized in the mitochondria which raises the intracellular ATP:Mg-ADP ratio. This leads to closure of the ATP-sensitive K_{ATP} channel which causes depolarization of the β -cell membrane. This activates voltage-gated calcium channels leading to an influx of Ca^{2+} which triggers insulin exocytosis.

13.3 Glucose Transporter 2 (GLUT 2) and Fanconi–Bickel Syndrome

GLUT2 is a transmembrane carrier protein enabling passive movement of glucose across cell membranes. Although widely expressed (liver, hypothalamus, small intestine, renal tubular cells) its main focus here is as one of three (GLUT 1, 2, 3) transporters of glucose into the pancreatic β -cell. Along with the other six members of the family of glucose transporter proteins (GLUT1–GLUT7), GLUT2 employs a facilitative transport mechanism – glucose is transported passively down a concentration gradient and, in contrast to ‘active’ transport, is energy *independent* [113]. Other features in common with its facilitative glucose transporter family are the 12 transmembrane components of GLUT2 and the fact that extracellular glucose binding induces a conformational change in the protein leading to intracellular release of glucose (Fig. 13.1) [90]. Thus GLUT2 demonstrates substrate specificity and saturation kinetics and is therefore particularly vulnerable to mutations affecting its transmembrane structure.

Although initially thought to be *the* glucose sensor of the cell, in reality the amount of extracellular glucose taken up by the β -cell bears no relation to the amount actually metabolized by the cell [24]. Whereas phosphorylation by glucokinase imposes a rate-limiting step on glycolysis in the β -cell (see Section 13.4), transport into the cell by GLUT2 is a highly efficient system due to its high $V_{\max}$ and K_M for glucose [44] which therefore provides an unrestricted supply of glucose for metabolism in the β -cell.

GLUT2 was first isolated from human liver and kidney cDNA libraries [35] and subsequently also localized to the rat pancreatic β -cell [91]. The human pancreatic islet identical counterpart was demonstrated in the same year [96] and the architecture of the 11 exon gene (*SLC2A2* also referred to as *GLUT2*) that encodes this glucose transporter on chromosome 3 was defined by Takeda et al. [125].

The importance of GLUT2 in carbohydrate metabolism is illustrated by the rare glycogen storage disease, Fanconi–Bickel syndrome (FBS) caused by inactivating homozygous mutations within *SLC2A2*. FBS is a rare autosomal recessive disorder, first described in 1949 [28], whose clinical features include hepatomegaly secondary to glycogen accumulation, glucose and galactose intolerance, fasting hypoglycaemia, a characteristic proximal tubular nephropathy and severe short stature [113]. This phenotype, characterized by glycogen excess in liver and kidney cells, emphasizes the role of GLUT2 in glucose output as well as uptake: in its absence, glucose produced from gluconeogenesis in the liver, and from tubular reabsorption in renal cells, is trapped and therefore stored as excess glycogen. Interestingly loss of *GLUT2* function in humans does not affect insulin secretion significantly [69]. In contrast *Glut2*-null mice display a lethal diabetic phenotype [46]. This is probably explained by the difference in the extent of *GLUT2* expression, most markedly in the pancreas, between rodents and man [19]. In addition there

may be another compensatory transporter activated on loss of *GLUT2* in human pancreatic β -cells, but this requires further investigation [69].

Treatment of FBS is symptomatic and includes a low-sugar, galactose-restricted diet, frequent small meals and replacement of fluids and electrolytes [113]. Thus FBS, and this naturally occurring loss of the principal pancreatic β -cell transporter system, is interesting in its manifestation as a glycogen storage disease (as all other diseases in that group are caused by enzymatic defects of glycogenolysis) rather than as a more typical diabetic phenotype.

13.4 Glucokinase and Defects in Glucose Homeostasis

Glucokinase (GCK) catalyses the first rate-limiting step in glucose metabolism through its phosphorylation of glucose on carbon 6 to form G6P. It is a member of the hexokinase family of 4 enzymes and recognized as the pancreatic β -cell ‘glucose sensor’ as its kinetics allow rate of glucose phosphorylation to vary over a range of physiological glucose concentrations (4–15 mmol/l) [74]. GCK has a lower affinity for glucose than the other three hexokinases and its activity is localized to fewer cell types (liver, pancreas, small intestine and brain); therefore the other hexokinases bear the brunt of glucose phosphorylation for glycolysis and glycogen synthesis in most tissues. The key distinguishing features of GCK which permit its specific function as glucose sensor in the β -cell are first its lower affinity for glucose than the other hexokinases and second the lack of inhibition by its product G6P which allows its continued stimulation of insulin release amid accumulating product [73].

GCK is encoded by the 12 exon gene *GCK* on chromosome 7 and consists of a monomeric protein of 465 amino acids. The crystal structure, only relatively recently defined in detail [61], vastly aids comprehension of the biochemical consequence of the various *GCK* mutations: there is a large and a small domain between which lies a deep cleft where glucose binds. As GCK binds glucose and ATP it undergoes a conformational change which approximates the large and small domains, resulting in a closed, active conformation. The GCK structure occurs in closed, open and ‘super-open’ conformations which define two catalytic cycles (slow and fast). The characteristic sigmoidal response of GCK to glucose is due to the ratio between these two catalytic cycles [61].

Genetically programmed defects in *GCK* include heterozygous inactivating mutations that cause a subtype of maturity-onset diabetes of the young (MODY), homozygous or compound heterozygous inactivating mutations that cause permanent neonatal diabetes mellitus (PNDM) and finally heterozygous activating mutations which cause hyperinsulinaemic hypoglycaemia [34, 38, 51, 88]. These defects in *GCK* all alter the efficiency of glucose binding and phosphorylation in the β -cell leading to increased or decreased glucose-stimulated insulin secretion and result in clinically appreciable hyperglycaemia or hypoglycaemia.

13.4.1 Mutations in Glucokinase (GCK) Cause Maturity-Onset Diabetes of the Young Subtype GCK (GCK-MODY) (Formerly Known as MODY 2)

MODY is thought to account for approximately 1–2% of diabetes [33, 68] and is an autosomal dominantly inherited form of diabetes characterized by young age of onset and pancreatic β -cell dysfunction. The first MODY gene to be identified was *GCK* [34, 51] and now over 600 mutations in *GCK* have been described worldwide [92]. All inactivating *GCK* mutations are associated with a mild fasting hyperglycaemia, the majority of patients having fasting blood glucose levels within a narrow range of 6–8 mmol/l, which is in contrast to all other forms of diabetes. This phenotype is perhaps to be expected given the fact that the heterozygous mutations simply cause reduced activity of the enzyme and a higher threshold for insulin release (on account of the compensation provided by the wild-type *GCK* allele) which is stimulated consistently nonetheless and accounts for the relatively benign natural history of GCK-MODY. Patients do not have accelerated deterioration of β -cell function and rarely need treatment – the majority being treated with diet alone; and perhaps most importantly in terms of morbidity and mortality in diabetes, patients with GCK-MODY rarely develop microvascular or macrovascular complications [25].

13.4.2 Permanent Neonatal Diabetes Mellitus due to GCK Mutations (GCK-PNDM)

In contrast to the mild phenotype described above, homozygous or compound inactivating *GCK* mutations lead to the severe condition of PNDM. This is a rare form of diabetes diagnosed within the first 6 months of life and homozygous mutations in *GCK* are now recognized as a rare cause of this condition. GCK-PNDM was first described in 2001 by Njolstad and colleagues and functional studies of the mutant *GCK* by the same group showed the enzyme's activity to be below 0.2% of that of the wild type [88]. To date only eight isolated cases of PNDM due to *GCK* mutations have been reported, either homozygous or compound heterozygous for a missense, frameshift or nonsense mutation leading to complete absence of glucokinase activity [87, 88, 98, 109, 129]. The severity of GCK-PNDM may vary depending on the amount of activity retained by the mutant enzyme [98]: the missense R397L mutation gives rise to a milder phenotype with the mutated *GCK* still able to stimulate insulin release, although not enough to avoid the need for supplemental insulin. All GCK-PNDM patients have required treatment with insulin for their diabetes although there is promising evidence in at least one case for the use of sulphonylureas (in addition to insulin) to augment improved glycaemic control [129].

13.4.3 Hyperinsulinaemic Hypoglycaemia due to GCK Mutations (GCK-HH)

Heterozygous gain-of-function mutations in *GCK* increase the affinity for binding glucose effectively reducing the threshold concentration of glucose that triggers insulin release, thereby leading to inappropriate over-secretion of insulin despite hypoglycaemia. The ensuing condition is hyperinsulinaemic hypoglycaemia and is also known as persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI), hyperinsulinaemia of infancy (HI) and congenital hyperinsulinaemia of infancy (CHI). Although the most common genetic cause for HH is mutations in the potassium channel genes (see Section 13.6), 12 causal activating mutations in *GCK* have been reported to date [7, 16, 17, 18, 38, 39, 75, 114, 131]. Interestingly the vast majority of these mutations occur in an allosteric activator site where small synthetic molecular activators which are currently under development for the treatment of T2D bind [16, 39, 45, 92].

The severity of hypoglycaemia in GCK-HH depends on the specific mutation: some cause possibly fatal episodes but the majority result in mild asymptomatic hypoglycaemia [92]. Treatment is by taking regular small meals but most require pharmacological intervention with the potassium channel activator diazoxide [39, 114, 131] or partial pancreatectomy.

The spectrum of clinical phenotypes ranging from hyperglycaemia to severe hypoglycaemia caused by genetically programmed defects in *GCK* in the pancreatic β -cell has given considerable insight into the workings of the system for glucose-stimulated insulin secretion here. In addition the functional characterizations of these inactivating and activating mutations have increased our knowledge of the mechanics of biochemical activation, structure and regulation of *GCK* which thus shows promise as a drug target for the treatment of common multifactorial T2D as well as these more rare monogenic β -cell dysfunction disorders.

13.5 Mitochondrial Mutations Impairing β -Cell Function and Mitochondrial Diabetes and Deafness (MIDD)

Mitochondria are membrane-bound organelles found in most eukaryotic cells and their key role is in producing the majority of a cell's chemical energy in the form of ATP. Mitochondrial dysfunction results in reduced oxidative phosphorylation and ATP synthesis and has most pronounced effect in high energy consuming tissues such as muscle, nerves and the pancreatic β -cell. They are unique in their genetics and inheritance: they carry their own circular DNA that contains 37 genes and inheritance is exclusively maternal as mitochondrial DNA is present in oocytes but not in spermatozoa.

Mutations in mitochondrial DNA are a rare cause of β -cell dysfunction accounting for 0.5–3% diabetes [64, 70, 81, 82, 136]. By far the most common mutation to

cause β -cell dysfunction and diabetes occurs in the gene that encodes tRNA leucine, leading to substitution of guanine for adenine (A $\rightarrow$ G) at position 3243 [43]. This mt.3243 A>G mutation leads to self-dimerization of the tRNA_{Leu} molecule causing impaired amino acid delivery to the ribosome and reduced protein synthesis: this leads to a reduction in oxidative phosphorylation and ensuing β -cell dysfunction [139]. The tRNA Leu 3243 mutation was originally identified in patients with the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) [43] although diabetes is not actually part of this syndrome: this association was made later [60] hinting at the range of phenotypes associated with this mutation. This variation in phenotype is due to different heteroplasmy loads across tissues and between individuals: heteroplasmy is the variable expression of wild and mutant mitochondrial DNA, with severest phenotypes having highest levels of heteroplasmy [66]. The β -cell dysfunction and diabetes associated with the mt.3243 A>G mutation is known as maternally inherited diabetes and deafness (MIDD) and was first described in 1992 [130]. As the name suggests, key clinical features are presence of diabetes and deafness and a family history amongst maternal relatives. The organs involved are manifest as those with highest metabolic rate such as muscle, kidney, brain, retina, cochlea and endocrine pancreas. Treatment varies widely according to the organ affected but most MIDD patients, although initially treated with diet or oral hypoglycaemics, will require insulin within a couple of years of diagnosis of diabetes [81, 82].

13.6 The K_{ATP} Channel and Defects in Glucose Homeostasis

ATP-sensitive potassium (K_{ATP}) channels control potassium flux across cell membranes, thus determining membrane potential, and connect metabolism within the cell to electrical activity. Increased metabolism, and therefore intracellular ATP:Mg-ADP ratio, closes the K_{ATP} channel leading to membrane depolarization, and increased electrical activity which can trigger events including muscle contraction and hormone release. The role of the K_{ATP} channel in pancreatic β -cell function and insulin secretion was elucidated in 1984 [4] and the importance of its role here is illustrated by the fact that mutations in the genes encoding the various channel components result in a spectrum of hypo- and hyperglycaemia disorders including transient neonatal diabetes mellitus (TNDM), PNDM and HH [1, 6, 29, 40, 41, 128, 102].

The β -cell K_{ATP} channel is an octameric complex of four inner pore-forming Kir6.2 subunits and four regulating outer sulphonylurea receptor 1 (SUR1) subunits [117]. Kir6.2 is encoded by *KCNJ11* on chromosome 11 and consists of a single exon encoding this 390 amino acid protein [56]. The SUR1 subunit is encoded by the gene *ABCC8* which is interestingly only ~ 4.5 kb from *KCNJ11*: *ABCC8* is significantly larger consisting of 39 exons and spanning greater than 100 kb [1].

Genetically programmed defects in the Kir6.2 or SUR1 K_{ATP} channel subunits cause a range of clinical phenotypes most obviously demonstrating a relationship

between severity of phenotype and degree of membrane hyperpolarization [5, 41, 126]. *KCNJ11* mutations cause a spectrum of phenotypes ranging from TNDM and PNDM through to the severe syndrome of developmental delay, epilepsy and neonatal diabetes (DEND) [40, 41]. This variability of phenotype is thought to be due to a combination of variation in Kir6.2 expression across tissues, mutation severity and compensatory mechanisms [50, 56, 63, 118].

13.6.1 Neonatal Diabetes Mellitus Caused by KCNJ11 Mutations

KCNJ11 mutations most commonly manifest as PNDM accounting for up to 34% cases [40]. This is the more severe form of neonatal diabetes (compared to TNDM) where reduced insulin secretion results in lowered birth weight and hyperglycaemia and diabetes that persists beyond 12 months of age. The most common *KCNJ11* mutation, R201H, causes PNDM through a 40-fold lowering of ATP sensitivity of the K_{ATP} channel and prolonged opening leading to reduced insulin secretion [40]. The most severe phenotype, DEND, associated with *KCNJ11* mutations typically causes the greatest K_{ATP} channel ATP insensitivity [101]. Involvement of extra-pancreatic tissues in DEND, in contrast to other phenotypes, may be explained by the highly activating nature of the causal mutations [103, 104]. The mildest phenotype associated with *KCNJ11* mutations is TNDM where diabetes remission usually occurs within 3–6 months. Functional studies of three *KCNJ11* mutations (G53R, G53S and I182 V) showed approximately a fourfold reduction in K_{ATP} channel ATP sensitivity demonstrating they are functionally less severe than the R201H mutation which causes PNDM [40, 41].

13.6.2 Hyperinsulinaemic Hypoglycaemia Caused by KCNJ11 Mutations

The heterogeneity of HH is illustrated by the range of causal mutations. In addition to GCK-HH (Section 13.4), a total of 24 *KCNJ11* mutations have been reported [29]. These cause HH by severely reducing K_{ATP} channel activity in the β -cell membrane [84].

13.6.3 Neonatal Diabetes Mellitus Caused by ABCC8 Mutations

TNDM and PNDM are also caused by *ABCC8* mutations, affecting the SUR1 subunit of the K_{ATP} channel, which are found in approximately 27% of PNDM patients in whom no *KCNJ11* mutation is identified [27]. The underlying mechanism here is accentuation of the effect of Mg-ADP on the K_{ATP} channel resulting in β -cell hyperpolarization and inhibition of insulin secretion [5]. *ABCC8* mutations have also been identified as a rarer cause of the more severe phenotype of DEND and iDEND: the

F132L mutation has been shown to alter the gating of the K_{ATP} channel as well as the sensitivity to Mg-ADP causing prolonged opening and severe membrane hyperpolarization [101, 103, 104, 105].

13.6.4 Hyperinsulinaemic Hypoglycaemia Caused by ABCC8 Mutations

ABCC8 mutations are the most common cause of HH and over 150 mutations have been described [29]. They have been functionally divided into two classes [3]: class I refers to an absent resultant protein at the membrane surface and class II refers to a channel that is present but persistently closed. Class I mutations lead to reduced protein levels or faulty trafficking of the channel [127]; class II mutations prevent K_{ATP} channel activation by reducing channel stimulation by Mg-ADP [54]. Generally the class I mutations have a more severe phenotype than the class II mutations which may be milder due to a partial response to Mg-ADP [29].

13.6.5 The Underlying Molecular Diagnosis in NDM and HH Has Implications for Treatment

The clinical implications of identifying an underlying K_{ATP} channel mutation in neonatal diabetes are significant given that many patients have now been successfully transferred from insulin to sulphonylurea therapy after the first confirmatory study in 2006 [94]. Even at the more severe end of the spectrum in DEND and iDEND some response to high-dose sulphonylurea therapy has been demonstrated [67, 79].

In contrast to GCK-HH, diazoxide is often not effective in HH due to *KCNJ11* and *ABCC8* mutations, which is perhaps to be expected given that the target for this drug is the K_{ATP} channel itself. Octreotide (a somatostatin analogue) has been used with some success in children [37]. Partial pancreatectomy is reserved for those who do not respond to medical treatment.

13.7 Defects in Glucose Homeostasis due to Mutations in Genes Encoding β -Cell Transcription Factors

Five of the eight causal MODY gene mutations occur in transcription factors [9, 32, 53, 72, 144]. The study of these naturally occurring mutations has increased our understanding of the genes and interlinking pathways required for normal function of the pancreatic β -cell. Hepatocyte nuclear factor 1 homeobox A (HNF1 α), hepatocyte nuclear factor 1 homeobox B (HNF1 β), hepatocyte nuclear factor 4 α (HNF4 α), neurogenic differentiation 1 (NeuroD1), insulin promoter factor 1 (IPF1) are all transcription factors regulating several genes in a tissue-specific

manner in the β -cell [78]. The precise underlying mechanism by which mutations in these transcription factors cause diabetes is unknown but a range of in vitro and in vivo studies, largely in rodents, has highlighted their importance in pancreatic β -cell development and differentiation as well as in the regulation of many genes involved in glucose-stimulated insulin secretion [12, 13, 83, 97, 133, 138, 143].

13.7.1 Mutations in Hepatocyte Nuclear Factor 1 Alpha (*HNF1 Alpha*) Cause Maturity-Onset Diabetes of the Young Subtype *HNF1A* (*HNF1A-MODY*) (Formerly Known as *MODY 3*)

HNF1 alpha was previously well known as a liver-specific transcription factor, but its role in diabetes pathophysiology and the pancreatic β -cell was uncovered after a genome-wide linkage scan [142]. *HNF1A* is encoded by a 10 exon gene on chromosome 12 and is a homeoprotein containing a DNA-binding domain through which the protein binds to its target DNA sequence as a dimer [15]. It has 90% amino acid homology in its DNA-binding domain with *HNF1B* and the two transcription factors bind to the same target DNA sequence [107]. *HNF1A* interacts with many other transcription factors and has several β -cell specific targets underlying its crucial role in normal β -cell function (Fig. 13.2): it shares a transcriptional feedback loop with *HNF4A* and, although *HNF1A* expression is restricted by *HNF4A* in hepatocytes, it is an upstream regulator in pancreatic β -cells [141]. In the β -cell *HNF1A* activates both the *GLUT2* gene and the L-type pyruvate kinase (*PKL*) gene (a rate-limiting enzyme of glycolysis) by binding to their promoter regions [134, 143]. *HNF1A* is also involved in the regulation of mitochondrial enzymes as well as organization of pancreatic islets through its regulation of E-cadherin, an adhesion molecule [141, 143]. Thus *HNF1A* has multiple roles in pancreatic β -cells (Fig. 13.2) perhaps making it a relatively common site of defects in diabetes subtypes.

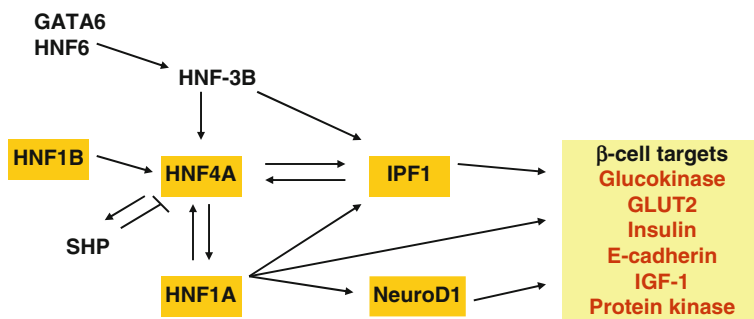


Fig. 13.2 The β -cell transcription factor network. The HNF network in pancreatic β -cells. *HNF4A* expression is mainly regulated by *HNF1A*. *HNF1B* functions with *HNF1A* as a homodimer or heterodimer. Transcription factors in orange boxes are known to be mutated in MODY subtypes.

Over 200 *HNF1A* mutations have been described with a common mutation at codon Pro291 (Pro291fsinsC) [26]: most mutations are localized along the DNA-binding, dimerization and transactivation domains of the protein [78]. *HNF1A* is the most commonly mutated gene in MODY accounting for approximately half of all cases [26]. Patients present in childhood or as young adults with deteriorating β -cell function over time and develop both microvascular and macrovascular complications. Patients respond well to sulphonylureas, which act downstream of many of the targets of HNF1A, and are the first-line choice of medication for HNF1A-MODY [95, 116].

13.7.2 Mutations in Hepatocyte Nuclear Factor 1 Beta (HNF1 Beta) Cause Maturity-Onset Diabetes of the Young Subtype HNF1B (HNF1B-MODY) (Formerly Known as MODY 5)

HNF-1 beta is another homeodomain containing transcription factor which functions as a homodimer or heterodimer with HNF1 alpha. Spontaneous mutations are not uncommon and heterozygous deletions on chromosome 17 encompassing the *HNF1B* gene account for around a third of known mutations [10]. It is thought that β -cell dysfunction due to *HNF1B* mutations is because of defects in pancreatic development [52]. The phenotype associated with *HNF1B* mutations also includes progressive nondiabetic renal dysfunction [86, 141] reflecting the high level of expression in the kidneys. Unlike the other MODY subtypes, patients with HNF1B-MODY are not sensitive to sulphonylureas and insulin treatment is most often required [25].

13.7.3 Mutations in Hepatocyte Nuclear Factor 4 Alpha (HNF4 Alpha) Cause Maturity-Onset Diabetes of the Young Subtype HNF4A (HNF4A-MODY) (Formerly Known as MODY 1)

The transcription factor HNF4 alpha is a member of the steroid hormone receptor family and binds to DNA as a homodimer. HNF4 alpha is thought to regulate similar pathways to HNF1 alpha which may be due to the fact that HNF4 alpha is a downstream regulator of HNF1 alpha in pancreatic β -cells, and a positive feedback loop involving both exists (Fig. 13.2) [141].

Mutations causing β -cell dysfunction occur in all exons and the pancreatic promoter [26] but are much less common than *HNF1A* mutations [112]. Clinical features associated are similar to HNF1A-MODY in adults but *HNF4A* mutations have also been found to account for a form of neonatal HH that resolves and later develops into MODY [62, 93], suggesting a differing role for these transcription factors in fetal and neonatal life. In addition to neonatal HH, *HNF4A* mutation carriers

are often macrosomic aiding distinction from HNF1A-MODY where neonates are generally of normal birth weight [93].

13.7.4 Mutations in Insulin Promoter Factor 1 (IPF1) Cause Maturity-Onset Diabetes of the Young Subtype IPF1 (IPF1-MODY) (Formerly Known as MODY 4)

IPF1 is another central transcription factor in the pancreatic β -cell where it regulates transcription of *GLUT2* and *GCK* and mediates glucose-stimulated insulin gene transcription as well as having a pivotal role in pancreatic development [59, 71, 89, 132]. Heterozygous *IPF1* mutations are a rare cause of MODY [14, 121] and homozygous and compound heterozygous mutations are a very rare cause of PNDM due to pancreatic agenesis [122]. More recently a novel *IPF1* mutation has been described where homozygosity was associated with a milder syndrome with only subclinical exocrine pancreas insufficiency [85].

13.7.5 Mutations in Neurogenic Differentiation 1 (NeuroD1) Cause Maturity-Onset Diabetes of the Young Subtype NEUROD1 (NEUROD1-MODY) (Formerly Known as MODY 6)

NeuroD1 is a transcription factor involved in regulating *GLUT2*, *GCK* and insulin gene transcription [65]. Mutations in *NEUROD1* have only been described in three families and are a very rare cause of MODY [72].

13.7.6 RFX6 Encodes β -Cell Transcription Factor Which When Mutated Causes Diabetes

Recently the transcription factor Rfx6 has been shown to be acting downstream of the transcription factor neurogenin 3 in the differentiation of pancreatic β -cells and formation of islets [120]. Autosomal recessive mutations in this gene are another cause of neonatal diabetes [77].

13.8 Mutations in Carboxy Ester Lipase (CEL) Cause Maturity-Onset Diabetes of the Young Subtype CEL (CEL-MODY)

Mutations in *CEL* have been described in individuals from families that conform to the MODY phenotype [106]; however, strictly speaking this is MODY due to a defect of the exocrine pancreas and not a defect in the pancreatic β -cell.

13.9 Endoplasmic Reticulum (ER) Stress as a Cause of β -Cell Death and Defects in Glucose Homeostasis

With the recent discovery of mutations in the insulin (*INS*) gene causing neonatal diabetes and subsequent functional studies to determine the molecular mechanism behind the mutations it is clear that ER stress plays an important role in pancreatic β -cell dysfunction.

13.9.1 Mutations in the Insulin (*INS*) Gene as a Cause of Neonatal Diabetes and Maturity-Onset Diabetes of the Young

Insulin gene (*INS*) mutations have been identified as causing PNDM and rarely MODY [11, 23, 36, 76, 80, 123]. There are two distinct mutational mechanisms. Autosomal dominant mutations stop disulphide bond forming, thus preventing normal folding of proinsulin within the endoplasmic reticulum (ER) in the pancreatic β -cell. The ER is sensitive to accumulation of unfolded proteins and has a specific unfolded protein response (UPR) to alleviate this stress. Failure of the UPR to clear unfolded proteins results in β -cell apoptosis [145]. Treatment of patients with *INS* mutations is with insulin therapy in order to reduce endogenous insulin production and protect the ER from accumulation of unfolded insulin [123]. A recent study has demonstrated that different mutations result in the production of [13] proinsulin molecules with markedly different trafficking properties and effects on ER stress [76]. In contrast another study has shown that autosomal recessive mutations cause neonatal diabetes through reduced insulin biosynthesis [36].

This model of accumulating misfolded proinsulin causing ER stress is supported by the Akita mouse model, which is a mouse model of MODY that develops diabetes as a consequence of β -cell dysfunction [135]. In this model a tyrosine for cysteine substitution at position 96 (C96Y) causes production of abnormal proinsulin: this collects within the ER causing ER stress which leads to β -cell death [2, 146].

13.9.2 Wolfram and Wolcott–Rallison Syndromes

ER stress within the pancreatic β -cell is also thought to be the mechanism underlying the rare genetic conditions of Wolfram syndrome (WFS) and Wolcott–Rallison syndrome (WRS). WFS, also known as DIDMOAD, causes a syndrome that includes diabetes, optic atrophy and deafness: in WRS the main clinical features are diabetes, multiple epiphyseal dysplasia, osteopenia, mental retardation or developmental delay and hepatic and renal dysfunction. WRS is the most common cause of permanent neonatal diabetes in consanguineous pedigrees [111] and is due to mutations in *EIF2AK3* which encodes a protein kinase-like ER kinase (PERK) [20].

PERK is highly expressed in pancreatic β -cells and its potential role in detecting misfolded proteins is supported by the fact that PERK knockout mice develop pancreatic β -cell death due to increased ER stress [48, 49]. More recent studies have highlighted the importance of PERK in the regulation of pancreatic β -cell differentiation and suggest that mutations in *EIF2AK3* cause pancreatic deficiency because of its specific developmental requirement in the fetal and early neonatal period [55, 147]. Wolfram syndrome is due to mutations in *WFS1* which encodes another transmembrane protein [57, 124] and the *WFS1* knockout mice also develop β -cell death and diabetes due to ER stress [58, 108, 140].

13.10 Common Genetic Variants Associated with T2D in Genes Implicated in Monogenic Forms of β -Cell Dysfunction

Genome-wide association scans for T2D susceptibility have now revealed up to 20 robustly implicated novel genetic variants (see Chapter 12) [100]. Due to their established role in monogenic diabetes the main β -cell genes discussed above have been cross-examined for harbouring common variants that influence T2D susceptibility (as well as the rare penetrant mutations that cause the monogenic conditions described above). One of the first to be identified was the E23K variant of *KCNJ11* [31, 39]. The underlying causal molecular mechanisms linking this variant to diabetes pathophysiology has yet to be defined precisely but recent functional studies have demonstrated the complexity of translating association signals to clear mutational mechanisms [47]. This illustrates the difference between rare mutations which have a large effect on protein function and common genetic variants which have more subtle effects. A variant within the *GCK* islet promoter [137] and another in LD with this (rs4607517) have been identified associating with fasting plasma glucose levels in the general population [99]. Most recently the variant rs11920090 in *SLC2A2* (encoding GLUT2) has been associated with fasting hyperglycemia [21]. These observations demonstrate that critical components of the pancreatic β -cell can exert their effects over an entire allelic spectrum with the functional severity of the defect dictating the clinical phenotype.

13.11 Summary

Figure 13.1 is a simple representation of the main components of β -cell glucose-stimulated insulin secretion. However, the variety of mutational mechanisms affecting specific components and the associated distinct phenotypes spanning severe hypoglycaemia, through to mild and then severe hyperglycaemia, illustrate a more complex interplay of biochemical pathways and structures. The study of these naturally occurring β -cell mutations has given much to our understanding of the mechanics of this system and in particular is providing novel drug targets. An example of this is the insight into GCK structure from GCK-HH mutations which have

highlighted the allosteric activator sight as a novel target for therapeutics aimed at attenuating GCK activity. The variation in phenotype encompassing both mild and severe manifestations of disease despite identical β -cell components affected also highlights the existence of compensatory mechanisms at work which have yet to be defined. The pancreatic β -cell is unique in its efficient translation of extracellular glucose to an individual's required level of insulin secretion: by the identification and study of a growing number of mutations with β -cell-specific effects, we are appreciating the complexity behind this system.

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Chapter 14

Proteomic Analysis of the Pancreatic Islet β -Cell Secretory Granule: Current Understanding and Future Opportunities

Garth J.S. Cooper

Abstract The pancreatic islet β -cell granule has been the subject of intense study for decades, in part because it serves as the vehicle for the regulated secretion of insulin and amylin, through which it exerts regulation of metabolism. β -cell granule proteins have been closely linked to disease mechanisms in both major types of diabetes, and recent findings from genome-wide association studies have reinforced the importance of these linkages for understanding disease mechanisms. Granule proteins have also proven to be of major interest in pharmaceuticals, since two of them, insulin and amylin, have each served as the basis for the development of anti-diabetic pharmacotherapies. In spite of all the attention this enigmatic granule has received to date, many fundamental questions about its molecular structure and function remain unanswered. In the past few years, high-resolution methodologies have begun to unravel the granule proteome in ever-increasing detail. Emerging data complement the results from the other approaches that have been applied to understand the granule. This chapter will explore the current state of knowledge in the field and the implications of emerging proteomic data for the study of physiological processes and disease mechanisms in diabetes.

Keywords Diabetes · Insulin granule · Amylin · Insulin · Proteomics · Post-translational · Modifications · β -cell degeneration · Cytotoxic protein aggregates · Autoantigens · Chaperones

Non-standard Abbreviations

2DGE	two-dimensional gel electrophoresis
AFM	atomic force microscopy

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ER	endoplasmic reticulum
FasL	Fas ligand
FADD	Fas-associated death domain protein
GAD	glutamate decarboxylase
HSP	heat-shock protein
ICA	islet-cell antigen
iTRAQ	isobaric tags for relative and absolute quantitation
MALDI-TOF	matrix-assisted laser-desorption-ionization time-of-flight
MS	mass spectrometry
MuDPIT	multi-dimensional protein identification technology
NAADP	nicotinic acid adenine dinucleotide phosphate
PC1	proprotein convertase 1
PTM	post-translational modification
RyR	ryanodine receptor
T1DM	type-1 diabetes mellitus
T2DM	type-2 diabetes mellitus
VAMP	vesicle-associated membrane protein

14.1 Introduction: Proteomics and the β -Cell Secretory Granule

14.1.1 *Proteomes and Proteomics: Definitions*

The proteome may be defined as the complete set of proteins expressed by a genome, cell, tissue or organism. Proteomes typically vary according to developmental stage and in response to environmental or genetic influences.

Subproteomes may also be defined. They may, for example, comprise all the metal-binding proteins, the phospho-proteins, the glycosylated proteins or the membrane proteins expressed by an organism, organ, tissue, cell or organelle, to mention a few of the myriad possibilities. There are clearly many different ways in which such subproteomes can be delineated.

Proteomics is the study of proteomes. Proteomic analysis frequently begins with the study of whole organs or tissues and then, according to the findings and emerging focus, proceeds to the examination of subcellular fractions or organelles, for example, the ‘mitochondrial proteome’, with increasing degrees of resolution [1]. Lipidomics, a complementary method, is a systems-based study of all lipids, the molecules with which they interact, and their function within a cell, tissue or organism. Proteomics is frequently employed in the first instance in its so-called ‘hypothesis-generating’ or ‘hypothesis-free’ mode, where it can be extremely effective in generating hypotheses, for example, by comparisons between related states [2]. Thereafter, it can be switched into its ‘hypothesis-driven’ mode, where hypotheses generated from analysis of the first phase of investigation can be explored in ever-increasing detail in a series of follow-up experimental designs.

Proteomic Analysis

Lipidomics

The genome describes the constant information that is passed on to be transferred from generation to generation in a type species and its cells. It contains all information needed for the assembly of all molecules to be produced by the cells. Different from that, the proteome is a dynamic feature that comprises all types of proteins present at a time and under some given conditions for an organism. The proteome does change depending on the stage of the life cycle and as a response to external conditions. Recently, also the term 'lipidome' was introduced to characterize the part of the metabolome that involves all types of lipids and lipid-like molecules in a cell, an organism or a tissue. Lipidomics is thus the science that studies the change of lipid profile depending on the life cycle stage or other parameters.

Further Reading

Watson AD (2006) Thematic review series: systems biology approaches to metabolic and cardiovascular disorders. Lipidomics: a global approach to lipid analysis in biological systems. *J Lipid Res* 47(10):2101–2111

Added by the editors

Amongst its many beneficial properties, proteomics is particularly adept at identifying and characterizing post-translational modifications (PTMs) in proteins [3, 4]. This ability to detect and quantify the contributions made to the modification of protein structure by the many possible post-translational modifications is now pointing the way towards levels of regulation in biological systems that are far more complex than has previously been envisaged [5, 6]. The multiple, regulated glycoisoforms of the protein adiponectin and the variable oligomeric structures that they generate provide a useful example of the complexity that is increasingly being unveiled by the systematic application of proteomic PTM analysis [2, 7, 8]. In future, proteomic PTM investigation is expected to contribute substantively to our understanding of disease aetiopathogenesis and to deliver many new targets for research into disease mechanisms and the generation of experimental therapeutics [3].

14.1.2 Proteomic Methods: A Very Brief Overview

Previously, proteomic methods frequently employed two-dimensional gel electrophoresis (2DGE) to perform the required separation of complex mixtures of proteins, followed by multi-dimensional mass spectrometry and informatics for

protein identification. Although 2DGE-based methods can yield important information [9, 10], for example, through the detection and characterization of groups of closely related proteins that differ in the quantity or quality of their similar PTMs [4, 11], they are also subject to the many shortcomings imposed by the physical properties of the many classes of proteins that cannot be resolved adequately using gels (for example, those with very high or low molecular weights, membrane proteins, those with high or low pI values, fibrous, very large or crosslinked proteins such as those occurring in the ECM, and those of low abundance). These limitations mean that most proteins present in most proteomes are inaccessible to analysis by 2DGE-based methods. Therefore there has been an increasing shift towards liquid chromatography (LC) or ‘gel-free’ based separation methods applied to proteolytic digests of whole proteomes with subsequent fractionation and labelling, for example, those based on *multi-dimensional protein identification technology* (MuDPIT). These approaches have the additional advantage that, with the introduction of approaches such as *isobaric tags for relative and absolute quantitation* (iTRAQ), semi-quantitative comparisons between multiple related proteomes have become feasible (*for examples, see* [12, 13]).

14.1.3 The Islet β -Cell and Its Secretory Granule: Targets for Proteomics

What is the possible relevance of proteomics to the insulin secretory granule? The pancreatic islets play a key role in the regulation of metabolism through their regulated secretion of the peptide hormones insulin, amylin and glucagon. They are of fundamental interest in the study of a broad range of disease mechanisms, including those characterized by dysregulation of pancreatic hormones as well as by disorders of hormone action, such as occur in insulin-resistant states [14].

Proteomic investigation of the pancreas has been motivated by several objectives. One of these is to improve our understanding of the mechanisms of islet hormone production and release and their linkages to the regulation of fuel metabolism [14]. Another is to identify proteins and, through them, processes that might provide better understanding of diseases that directly impact on the pancreatic tissues, chief amongst which are diabetes mellitus, pancreatitis and pancreatic cancer [15–22].

At one level, investigation of the pancreatic proteome has arguably been underway for most of the last hundred years, driven in large part by the need to understand and reverse the processes that lead to or cause diabetes. Islet amyloid or ‘hyaline’ (Fig. 14.1) was the original observation that linked degeneration of the islets of Langerhans to the causation of the form of the disease now known as type-2 diabetes (T2DM) [23, 24]. Fundamentally important results from early protein chemical studies of the pancreas led to the isolation and characterization of insulin and the development of insulin therapy, initially for type-1 diabetes (T1DM) and later for T2DM [25].

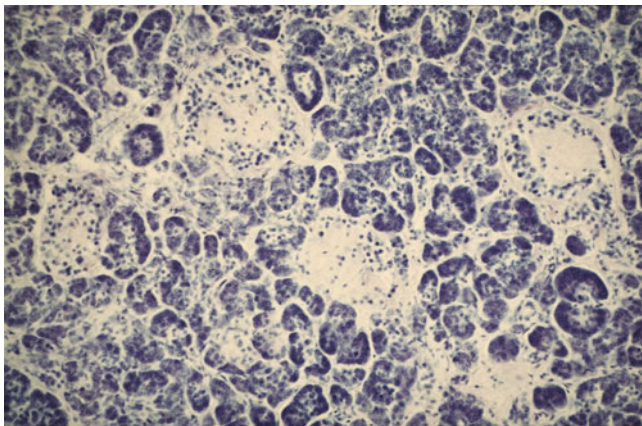


Fig. 14.1 Microscopic view of an original haematoxylin and eosin-stained pancreatic section wherein islet ‘hyaline’ (now known as amyloid) was first identified by Dr. Eugene Opie, in a post-mortem study of a patient with type-2 diabetes mellitus [23]. The islets, which are usually replete with endocrine cells, have largely been replaced by the amorphous, faintly pink-staining islet amyloid. (Reproduced with permission of Robert D. Hoffman, M.D., Ph.D., Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD).

Decades later, granule proteins were identified as likely targets of immune mechanisms related to the aetiology and pathogenesis of T1DM [26].

14.1.4 Granule-Associated Pathogenic Processes and the Origins of Diabetes

Increasing evidence has implicated misfolding of the β -cell hormone amylin [27–29] to generate cytotoxic oligomers [30, 31], as potentially responsible for β -cell degeneration in T2DM [32]. These phenomena provide a clear rationale for elucidation of the composition of the β -cell granule at high resolution, with the aim of identifying intrinsic molecular pathways that might mediate as-yet unknown granule functions [20, 22] – for example, those relating to the control of protein folding within the granule – and possible defects that might contribute to the formation of cytotoxic protein aggregates [20, 33].

Evidence underpinning facets of this emerging pathogenetic mechanism is developed in the following section, to provide an example of but one of the important unsolved mysteries of the β -cell secretory granule, which may prove amenable to proteomic study.

There are at least two other well-recognized pathobiological questions of fundamental importance relating to the granule, where proteomic analysis could also have a part to play.

14.1.5 Granule Proteins as Putative Autoantigens in T1DM

The first of these questions relates to the nature of the component proteins that act as autoantigens in the autoimmune destruction of islet β -cells, for example, in T1DM. Numbers of proteins have been identified as candidate autoantigens with potential relevance to the mechanisms of autoimmune β -cell destruction, either through studies in animal models such as the non-obese diabetic (NOD) mouse or in human patients [34–37]. Most are components of β -cell secretory granules, although they may also exist in other organelles, and frequently in other cell types as well. Several of these candidate autoantigens are recognized by T-lymphocytes, including insulin, glutamate decarboxylase (GAD) 65 and GAD 67, heat-shock protein 65 (HSP65) and islet-cell antigen 69 (ICA69) [38]. Nevertheless, there remains uncertainty concerning the nature of another group of autoantigens associated with the secretory granule [39]. Indeed, there is evidence for recognition of novel islet T-cell antigens by β -cell granule-specific T-cell lines from new-onset T1DM patients, where a fraction of islet β -cells appear to be targeted predominantly by autoreactive T-cells [39].

These considerations point to a need for improved understanding of the protein components of the secretory granule for the following reasons: (i) to identify new potential autoantigens and (ii) to better elucidate the mechanisms that evoke T-cell activation and T-cell-mediated autoimmune β -cell destruction in T1DM.

14.1.6 Granule Proteins and Hormone Secretion

The second question relates to defective insulin secretion in T2DM. B-cell secretory granules have been studied for decades, with the major aim of elucidating the mechanisms of insulin processing and secretion [40–42]. Recent results from genome-wide association studies (GWAS) [43] have reinforced linkages between defective insulin secretion, β -cell granule proteins and the pathogenesis of T2DM [44, 45].

Many studies of the role of β -cell granules in insulin secretion have focussed on the mechanisms by which insulin is processed and stored [41, 42] and the roles of ion channels [46–48] and other proteins in the regulation of their exocytosis [49–58]. Some of the proteins that mediate exocytosis, for example, the small Rab GTPases and VAMP2, are intrinsic to substructures within the β -cell secretory granule [59, 60], whereas others reside in other parts of the cell and may therefore not co-purify with granules.

In recent years, increasing evidence has pointed to the secretory granule itself playing a leading role in the triggering of its own secretion. In particular, ryanodine receptor (RyR) I-mediated Ca^{2+} -induced Ca^{2+} release from the β -cell secretory granule, possibly potentiated by nicotinic acid adenine dinucleotide phosphate (NAADP), is increasingly seen to play an essential role in the activation of insulin secretion [61, 62]. Receptors for NAADP, a novel intracellular Ca^{2+} -mobilizing agent [63, 64], may represent an alternative pathway for Ca^{2+} efflux from β -cell

secretory granules [65]. Islets and MIN6 β -cells express two RyR isoforms, RyR I and RyR II, which display distinct subcellular localizations. Whereas type-I RyRs were present in approximately equal density in a mixed vesicle/mitochondrial fraction and in microsomes, RyR II was considerably more abundant on ER membranes [62]. Functional NAADP-sensitive Ca^{2+} stores are also present in human β -cells [66].

Dantrolene, a selective inhibitor of RyR I, increased steady-state free $[\text{Ca}^{2+}]$ in β -cell secretory granules but not in the ER, consistent with the presence on granules of a further activator or channel capable of amplifying the effects of RyRs on Ca^{2+} release. Receptors for NAADP may thus serve this role, and insulin secretory vesicles, but not the ER, may comprise an NAADP-responsive Ca^{2+} store [62]. Regulated calcium storage in the insulin secretory granule has thus been implicated in the mechanisms of regulated insulin secretion.

This emerging picture implicates Ca^{2+} regulation within the insulin secretory vesicle itself as pivotal to the regulated secretion of insulin and amylin and points to areas where proteomic analysis might be able to contribute to the elucidation of molecular mechanisms. Questions that arise include those of the nature of the proteins that might mediate aspects of this emerging process, the nature and roles of putative Ca^{2+} -binding proteins in the insulin secretory granule and, ultimately, the nature of the molecular defects in T2DM that generate defective insulin secretion.

In order to understand such processes at the molecular level, it would help to know which proteins and pathways are actually present in the granule, so as to understand which may possibly be implicated in disease processes that might occur therein.

14.1.7 Potential Future Contributions by Proteomics

Phenomena such as those which potentially link aspects of insulin secretory granule function to the misfolding of amylin, the regulation of insulin and amylin secretion, the generation of β -cell autoantigens and through these processes to the pathogenesis of the major types of diabetes provide a clear motivation and focus for the systematic, ongoing investigation of this organelle.

Proteomic analysis is but one of a number of hypothesis-generating methodologies that are now being brought to bear on questions concerning the origins and mechanisms of diabetes and the roles of granule-associated pathways in these processes. One of its advantages in this case is that it can be selectively targeted at the granule itself, as explained below. Other hypothesis-generating methods include GWAS, whole-genome transcriptomics and metabolomics, which together contribute different aspects of the information available in the broader field of systems biology and are broader but less focussed in their scope.

One of the challenges that will need to be met in the next phase of the application of systems biology to the insulin secretory granule is the integration and interpretation of the large data sets currently being generated by these complementary but distinct methodologies, with the objective of generating testable hypotheses for the

targeted dissection of disease mechanisms and the use of these in the generation of new, integrated hypotheses whose final goal must be the generation of new and improved therapeutic interventions for diabetes.

14.2 β -Cell Secretory Granules: Structural Regions and Functional Specialization

The β -cell granule performs a specialized subcellular function in the storage and secretion of insulin and amylin. It is a complex intracellular organelle containing many proteins with different catalytic activities and messenger functions [24, 67, 68] along with other components including adenine nucleotides, inorganic phosphate and bivalent metal ions [69].

The granule itself comprises several distinct structural regions, including the dense core with its component insulin- and zinc-containing crystals, the halo, and the enveloping, VAMP-containing outer membrane [60, 68, 70–72]. There is evidence for differential distribution of component proteins between these different regions, which subserve distinct functions. These different structures could possibly be targeted individually in future proteomic studies, with consequent increases in resolution and improved understanding of protein distribution and function within the granule.

B-cell secretory granules can be visualized by electron microscopy as spheroidal structures of about 200–300 nm in diameter (Fig. 14.2) and comprise a crystalline core of zinc/insulin-containing crystals surrounded by a mantle of less dense material, enwrapped by a phospholipid bilayer membrane [68, 69]. The granule is, however, far more than just a cellular repository for processed insulin. For example,

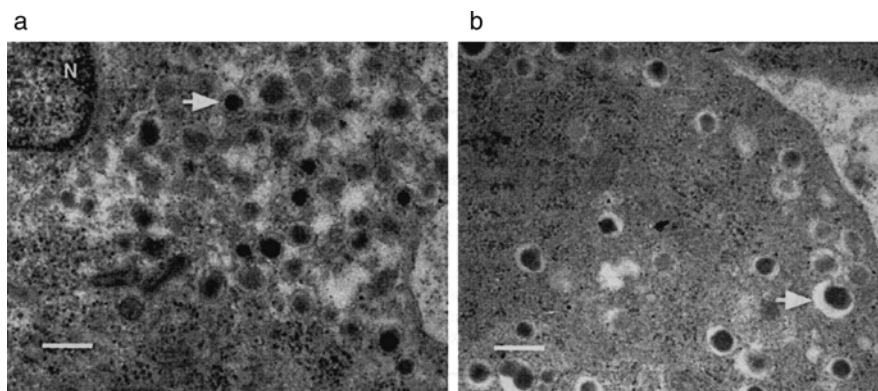


Fig. 14.2 Electron micrographs illustrating secretory granules from cultured murine insulin-secreting β C6-F7 cells. (A) Structures in the perinuclear region show characteristic membrane-limited granules in different stages of maturation (*arrowed*). (B) Mature secretory granules near the cellular periphery show characteristic electron-dense cores and adjacent electron-lucent haloes (*arrowed*). N, nucleus; Bars = 300 nm. (Reproduced with permission from [114]).

its membrane contains a series of proteins involved in the integration of its trafficking and docking to the cell membrane and (as discussed above) it contributes actively to its own secretion through regulation of cell- Ca^{2+} metabolism [64].

In the early 1980s, Hutton reported that β -cell granules may contain more than 150 distinguishable proteins in addition to their major constituents, which were considered to be insulin and its connecting peptide (C-peptide) [73], and that a number of these are secreted in addition to insulin [74]. Additional granule components were noted to include proteinases implicated in proinsulin-to-insulin conversion, intermediates in that conversion process, minor co-secreted peptides, membrane proteins that mediate granule movement and exocytosis, and ion-translocating proteins involved in the regulation of the within-granule environment.

More recently, amylin (designated also as IAPP) was found to be a second major hormone packaged in the β -cell secretory granules, which is also mainly β -cell-specific [27, 75]. Interestingly, amylin may be predominantly present in the granule haloes, whereas processed insulin resides mainly in the dense cores.

Typical β -cells contain about 10^4 insulin secretory granules, but less than 1% of these are thought to be available for immediate release [71]. All the rest are considered to be immature and must be primed and then recruited to the cell membrane before they can undergo exocytosis. These processes require several ATP-, Ca^{2+} - and phosphatidylinositol-4,5-bisphosphate ($\text{PI}(4,5)\text{P}_2$)-dependent steps, which culminate in pore formation and granule release to the cell exterior [70, 76].

14.2.1 Before Proteomics: Major Protein Components of the β -Cell Secretory Granule

The protein composition of the endocrine pancreas has arguably been under investigation for most of the past 100 years. The initial impetus was provided by the search for the hypoglycaemic principle, which turned out to be insulin [25]. For many years after its discovery, until the advent of recombinant human insulin manufactured in microbial expression systems, bovine and porcine pancreases were the only feasible sources of insulin for clinical use [77]. The extraction of pharmacologically active insulin from the pancreas, wherein the predominant exocrine cells are replete with proteolytic hormones, was said to be one of the most challenging of all extractions of natural products for pharmaceutical purposes [77].

The considerable heterogeneity of highly purified insulin preparations was demonstrated by application of various chromatographic and electrophoretic methods well before the discovery of proinsulin. Biochemical analysis of the pancreatic islets subsequently yielded the insulin precursor, proinsulin [42, 78, 79], and with it in time the understanding of insulin release by enzyme-catalysed conversion from proinsulin [42].

These pre-proteomic era studies provided a platform on which current proteomic analysis of the β -cell secretory granule may be anchored. Modern proteomic studies are thus seen as an extension of this earlier work.

14.3 Evolution of a Question That Might be Addressed by Proteomics: 'How Might Amylin Misfolding Cause T2DM?'

Islet amyloid is formed mainly by misfolded human amylin [27], a physiological resident of the islet β -cell granule (Figs. 14.1 and 14.3). Aggregation of the human hormone into small soluble β -sheet-containing oligomers is linked to islet β -cell degeneration and the pathogenesis of T2DM [31–33]. Islet amyloid is associated

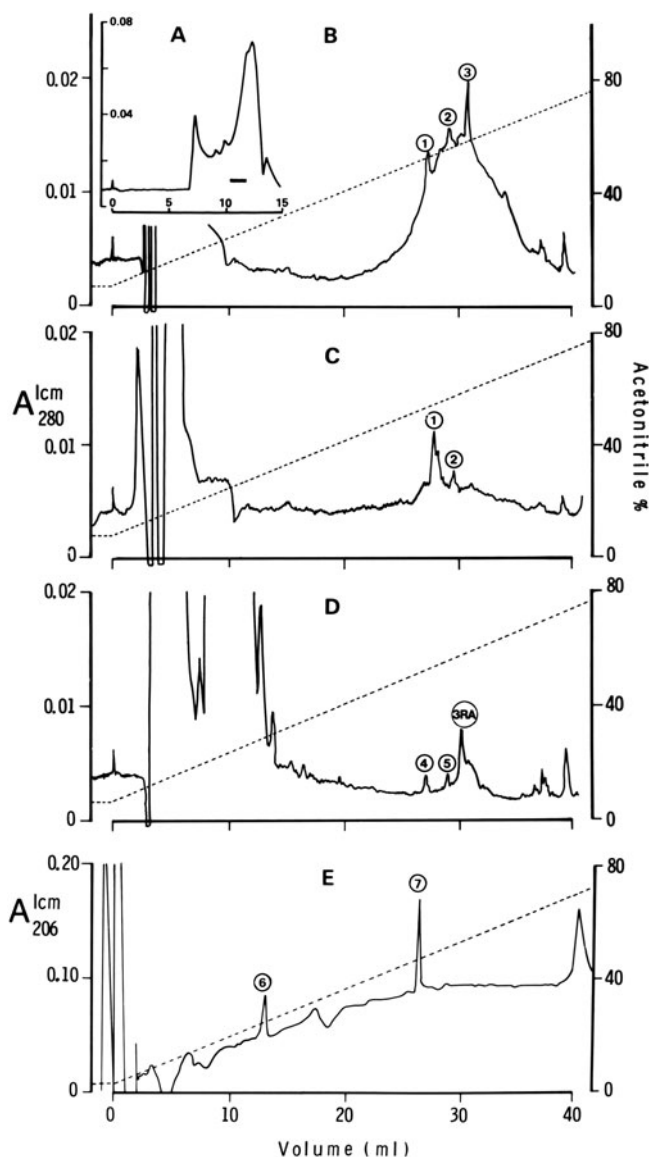


Fig. 14.3 (continued)

with substantial reductions in relative β -cell mass in type-2 diabetes (on average $\sim 60\%$), probably due to increased apoptosis compared with obese and lean non-diabetic humans [80].

Several lines of evidence now provide compelling support for the idea that processes associated with amylin aggregation contribute to β -cell degeneration and the onset of T2DM.

First, in vitro studies with synthetic amylin show that fibrillar structures assemble spontaneously through self-association of monomers into protofibrils and higher-order fibrillar structures [81, 82]. Studies with time-dependent atomic force microscopy have enabled direct in vitro visualization of this process. These studies show that oligomer formation can take place within minutes [31], a time course that matches the activation of the β -cell membrane Fas/FasL/FADD-activated pathway in β -cells destined to undergo amylin-evoked apoptosis [33].

Cytotoxic amylin preparations contain few preformed fibrils, but undergo time-dependent aggregation into soluble β -conformers [83]. Islet β -cell toxicity evoked by aggregating extracellular amylin occurs through an apoptotic mechanism [84, 85] mediated via a pathway comprising initial activation of a membrane-bound Fas/FasL/FADD/caspase-8 complex [33, 86] followed by a three-pronged downstream cascade comprising JNK1/cJun [86], ATF2/p38 MAPK [87] and p53/p21WAF1/CIP1 [85], which results ultimately in activation of caspase-3 [86] and consequent apoptosis. In addition, parallel amylin-mediated activation of ER stress related pathways might also contribute to islet β -cell degeneration [88].

Second, associations between human amylin aggregation and decreased β -cell mass have been reported from in vivo studies in several murine transgenic models of amylin-mediated diabetes [29, 32, 89–93] (Fig. 14.5). Similar associations are present in primates, whose wild-type amylin molecules contain an amyloidogenic sequence [24, 94, 95], are fibrillogenic and form islet amyloid [96–99]. By contrast, murine amylin molecules are not aggregation-prone [100], so diabetic phenotypes in human amylin transgenic mice develop in a background devoid of amyloid formed by the wild-type murine hormone. Obese human amylin transgenic mice have been

Fig. 14.3 Original chromatographic purification of human amylin from pancreatic extracts of type-2 diabetic patients provides an early example of the application of comparative proteomic analysis to tissues. (A) HPLC gel filtration in 6 M guanidine hydrochloride of an extract from an amyloid-containing pancreas taken at *post-mortem* from a patient with type-2 diabetes. Amylin was present in the region indicated by the bar. (B) Reversed-phase HPLC of material from the region indicated by the bar in A: unreduced amylin was present in peak 3. (C) Reversed-phase HPLC of a control extract from a control pancreas from a non-diabetic patient, as in B. Peaks 1 and 2 corresponded in elution time and amino acid composition to 1 and 2 in B. (D) Re-purification by reversed-phase chromatography of peak 3 in B after reduction and alkylation of cysteine residues and [^{14}C]-radiolabelling of cysteinyl residues. Peaks 4 and 5 had amino acid compositions distinct from that of 3RA, which was reduced and alkylated amylin. (E) Separation of product peptides after tryptic digestion of reduced and alkylated amylin by reversed-phase HPLC. Peak 6 was the smaller, more hydrophilic peptide amylin_{1–11}, and peak 7 the larger, more hydrophobic amylin_{12–37}. All radiolabel was present in peak 6. Identity of peaks was confirmed by quantitative amino acid analysis and by gas-phase peptide sequencing. The ratio of peak heights is consistent with the relative lengths of the amylin-derived peptides. (Reproduced with permission from [27]).

reported to replicate pathological findings in T2DM, showing non-ketotic hyperglycaemia, amyloid deposition and decreased β -cell mass, possibly via increased apoptosis [92].

Amylin aggregation could thus mediate β -cell degeneration in T2DM. However, the significance of tissue aggregates comprising mature amyloid fibrils in T2DM pathogenesis remains uncertain, since some studies have implied that amyloid fibrils themselves may be toxic or that human amylin transgenic mice may not develop spontaneous diabetes [91, 101–104]. The latter discrepancies may well be explained, however, by a requirement for permissive genetic background–human amylin transgene interactions to manifest full-blown β -cell degeneration and a diabetic phenotype [32, 93, 105].

Finally, human amylin purified from islet amyloid deposits is a potent inducer of insulin resistance in ex vivo rat skeletal muscle [106, 107] (Fig. 14.6). This finding provides a potential link between islet β -cell dysfunction and the induction of peripheral insulin resistance.

These observations, and the ongoing uncertainty concerning precise mechanistic linkages between amylin aggregation, β -cell degeneration, the regulation of systemic fuel metabolism and T2DM onset [108], provide fertile ground for future proteomic investigation. For example, the exact location and mechanism of amylin misfolding is unknown, as is the site of origin and nature of the amylin-mediated death-initiating signal – is it cell membrane bound Fas/FasL/FADD activation [33], ER stress [88] or some other process that might occur elsewhere [109, 110]?

One key question that is yet to be answered is whether amylin-mediated misfolding occurs prior to, within or after amylin secretion from the pancreatic islet β -cell granule. Granule-focussed studies are expected to prove crucial in answering this key question.

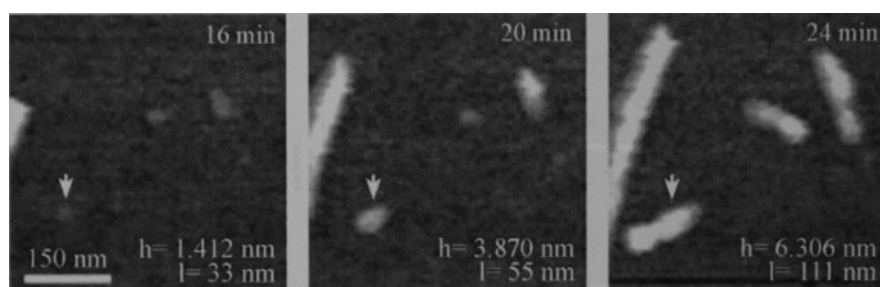


Fig. 14.4 Time-lapse atomic force microscopy (AFM) showing a human amylin oligomer growing into a fibril. Droplets of a human amylin solution were placed on a mica surface and studied by AFM using published methods [31]. Oligomers are seen to grow in height prior to extensive elongation into fibrils and consist of ~ 16 monomers when first visualized (*left-hand panel*). The height of the oligomer (*arrow*) is seen to increase with each scan. The time points and height (h) and length (l) measurements are as indicated in each image. (Reproduced with permission from [31]).

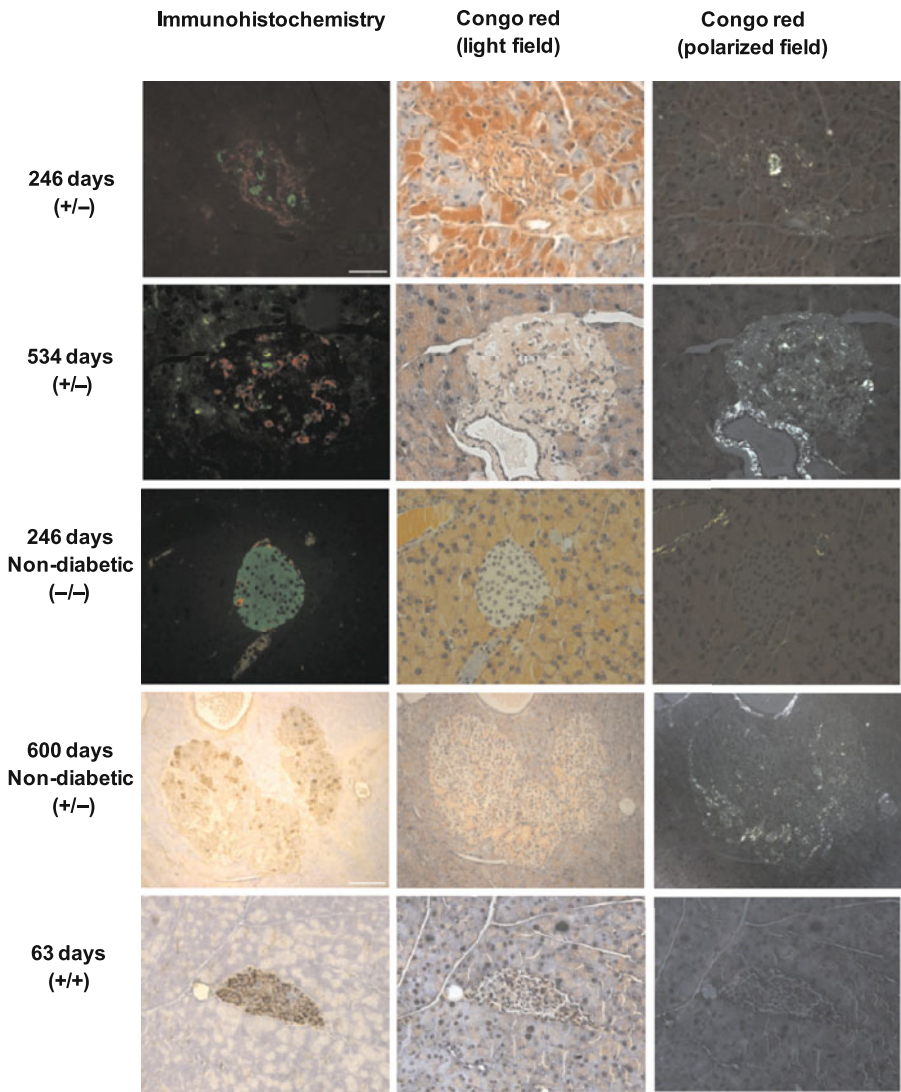


Fig. 14.5 Amyloid visualized by light microscopy was dissociable from occurrence of diabetes in hemizygous human amylin transgenic mice. Photomicrographs show serial pancreatic islet sections from non-transgenic and human amylin transgenic animals. *Left photomicrographs from top three panels* show insulin (green) and glucagon (red) immunoreactivity. *Bottom two left panels* show islet sections incubated with antisera to somatostatin and glucagon, revealing brown cytoplasmic staining. *Middle and right panels* show corresponding light- and polarized-microscopic field views of adjacent islet sections stained with Congo red. Amyloid birefringence is *apple green* whereas that corresponding to collagen is *silvery*. The scale bar (50 μ m) shown in top left photomicrograph applies to all images except for those corresponding to the 600-day non-diabetic hemizygous mouse (*second to bottom row*) which represents 100 μ m. (Reproduced with permission from [32]).

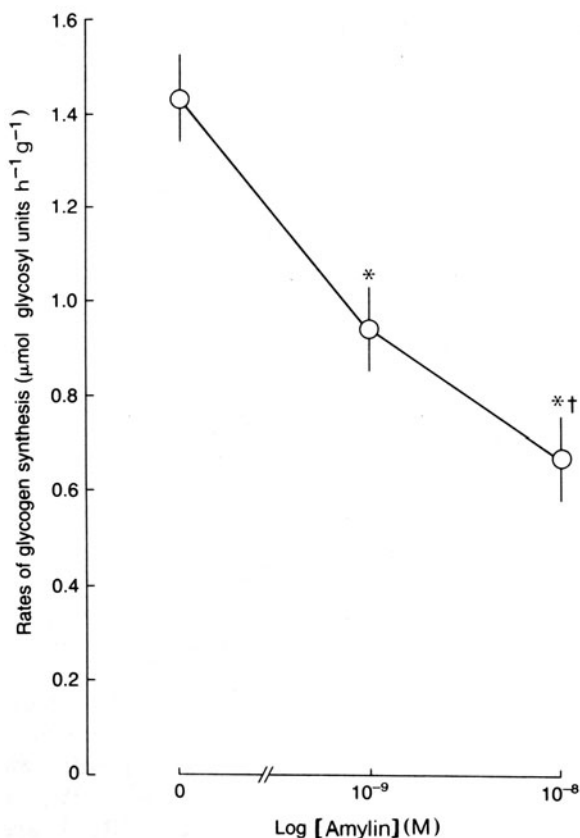


Fig. 14.6 Human amylin extracted from the pancreas of type-2 diabetic patients elicits dose-dependent inhibition of insulin-stimulated glycogen synthesis in ex vivo rat soleus muscle. The human amylin used in these studies was purified and characterized by the methods of Cooper et al. [27]. Values shown are means of at least four separate incubations. Statistically significant ($p < 0.05$, Student's *t*-test) decreases from control values are indicated by * (control against 10^{-9} M amylin); † (10^{-9} M amylin against 10^{-8} M amylin). Methods: Muscle preparation and incubation methods were as described [106]. Typically, a submaximal concentration of insulin ($100 \mu\text{U ml}^{-1}$) stimulates the rate of glycolysis and glycogen synthesis, by ~ 50 and 125% above basal rates, respectively, in this preparation. (Reproduced with permission from [106]).

14.4 The β -Cell Secretory Granule Proteome

Available proteomic studies of whole mouse [111] and human [112] islets have identified groups of islet-specific proteins. The bulk of proteins detected overlap with those in other tissue types, but islet hormones were also identified. The resulting peptide reference libraries are seen as providing a resource for future higher throughput and quantitative studies of islet biology, which may be useful in the study of T2DM mechanisms, for example.

Proteomics has been applied to analyse the effects of insulin signalling in isolated murine and human islets, in a study that provides a useful example of how this methodology can be integrated with other methods to address a complex research question – in this case, the role of insulin in β -cell apoptosis [113]. In this study, combined data, to which proteomic analysis made a significant contribution, indicated that insulin can act as a master regulator of islet survival by regulating Pdx1.

14.4.1 Proteomic Approach to the β -Cell Secretory Granule

Two systematic proteomic analyses of β -cell secretory granules have been reported in the past few years [20, 22]. Results from one of these are detailed here (Table 14.1) as a basis for the following comparisons and contrasts [20].

These studies reported the molecular identities of 51 and 130 ‘granule-related’ proteins [20, 22], respectively. In each case, most of the proteins reported were newly identified as potential granule components.

Without doubt, these findings usher in a new era of studies of the β -cell secretory granule. These studies point to numerous proteins and pathways that have not previously been identified in β -cell secretory granules and thus have the potential to greatly increase our knowledge of the intricate functionality of these organelles.

However, comparisons and contrasts between the two are illustrative of some of the challenges confronted by application of proteomics to the β -cell secretory granule at present, and caution is warranted in their interpretation and application. Intensive follow-up work is required before the status of each newly identified putative granule protein is confirmed and clarified.

Both groups analysed granules purified from insulin-secreting rat INS-1E cells, which came initially from the same source. Comparisons and contrasts between the two studies are therefore informative for several reasons, so they are now described in greater detail.

14.4.2 Method Comparisons

Both studies identified many proteins that had previously been associated with β -cell secretory granules by pre-proteomic methods, and there were considerable overlaps between the two data sets [20, 22].

However, Brunner et al. reported many proteins usually considered to be lysosome-associated, as components of their purified β -cell secretory granule proteome. By contrast, Hickey et al. described numbers of proteins more usually associated with ER and mitochondria, some of which did not appear in the data set of Brunner et al. These differences deserve further consideration, since they highlight potential challenges faced by current proteomic techniques.

Table 14.1 Proteins identified from immunopurified insulin granule preparation by LC-MS/MS

Protein	Location	Score ^a	NCBI accession number	[Peptides] ^{2+,3+} sequenced; sequence coverage	Function
Protein folding					
Heat-shock protein 5	ER	2834 648	gi 25742763	14; 27%	Involved in protein folding and assembling/disassembling of protein complexes
GRP58	ER	485	gi 38382858	12; 26%	Catalyzes the rearrangement of -S-S-bonds in proteins
Protein disulphide-isomerase A6	ER	430	gi 62296810	7; 22%	Catalyzes the rearrangement of -S-S-bonds in proteins
Tumour rejection antigen gp96	Cy	309	gi 58865966	5; 9%	Molecular chaperone. Has ATPase activity (by similarity)
dnaK-type molecular chaperone hsp72-ps1	?	277	gi 347019	5; 11%	Involved in protein folding and assembling/disassembling of protein complexes
Heat-shock protein 90-kDa protein 1, alpha	Cy	246	gi 51859516	4; 7%	Molecular chaperone. Has ATPase activity (by similarity)
Protein disulphide-isomerase	ER	134	gi 129731	3; 3%	Catalyzes the rearrangement of both intra- and inter-chain disulphide bonds in proteins to form the native structures
Hypoxia upregulated 1	ER	100	gi 77404375	2; 2%	Functions as a molecular chaperone in the endoplasmic reticulum for the folding and trafficking of newly synthesized proteins

Table 14.1 (continued)

Protein	Location	Score ^a	NCBI accession number	[Peptides] ^{2+,3+} sequenced; sequence coverage	Function
Peptidyl-prolyl cis-trans isomerase A	Cy	72	gi 118107	2; 17%	Accelerates the folding of proteins. Catalyzes the cis-trans isomerization of proline-imide peptide bonds in oligopeptides
Stress-70 protein	Mt	70	gi 116242506	2; 3%	Implicated in the control of cell proliferation and cellular aging. May also act as a chaperone
Chaperonin containing TCP1, subunit 3	Cy	63	gi 40018616	1; 2%	Chaperone
Energy metabolism					
ATP synthase (beta subunit)	– Mt	1828 417	– gi 149029718	– 10; 24%	Involved in oxidative phosphorylation
M2 pyruvate kinase	Mt	273	gi 206205	5; 11%	Involved in final step of glycolysis
ATP synthase (alpha subunit)	Mt	245	gi 40538742	5; 12%	ATP synthesis coupled proton transport
ATP citrate synthase (ATP citrate (pro-S)-lyase)	Mt	210	gi 113116	6; 7%	Involved in energy production and conversion
Acid alpha-glucosidase	Mem	157	gi 40018606	3; 4%	Carbohydrate transport and metabolism
Glyceraldehyde-3-phosphate dehydrogenase	Cy	133	gi 56188	3; 7%	Carbohydrate transport and metabolism
Aldolase A	Cy	115	gi 202837	3; 7%	Carbohydrate degradation and glycolysis
Aconitase 2	Mt	62	gi 38541404	2; 3%	Catalyzes the interconversion of citrate to isocitrate via cis-aconitate in the second step of the TCA cycle

Table 14.1 (continued)

Protein	Location	Score ^a	NCBI accession number	[Peptides] ^{2+,3+} sequenced; sequence coverage	Function
Protein kinase C substrate	ER	62	gi 149020437	1; 1%	Regulatory subunit of glucosidase II
Glutamate oxaloacetate transaminase 2	Mt	54	gi 6980972	1; 2%	Transaminase involved in amino acid metabolism and fatty acid transport
Alpha enolase	Cy	51	gi 56757324	1; 2%	Multifunctional enzyme that, as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses
Cytochrome c oxidase subunit 5B	Mt	49	gi 473729	1; 13%	A nuclear-encoded subunit of cytochrome c oxidase, the terminal oxidase in mitochondrial electron transport
Trafficking/exocytosis					
Tubulin (beta)	CS	1541 270	gi 224839	6; 16%	Major microtubule constituent that binds GTP
GTP-binding protein Rab1	Cy	214	gi 45433570	5; 26%	Rab subfamily of small GTPases implicated in vesicle trafficking
Beta actin	CS	207	gi 13592133	5; 14%	A ubiquitous protein involved in filament formation and a major cytoskeletal component
Tubulin (alpha 1A)	CS	198	gi 11560133	5; 15%	Major microtubule constituent that binds GTP
Alpha actin	CS	150	gi 9506371	3; 10%	A ubiquitous protein involved in filament formation and a major cytoskeletal component

Table 14.1 (continued)

Protein	Location	Score ^a	NCBI accession number	[Peptides] ^{2+,3+} sequenced; sequence coverage	Function
GTP-binding protein rab3A	Mem	123	gi 61098195	4; 17%	Regulates late synaptic vesicle fusion. May play a role in neurotransmitter release by regulating membrane flow at nerve termini. Interacts with RAB3IP
GTP-binding protein rab2A	ER	115	gi 13929006	2; 12%	Essential for protein transport from the ER to the Golgi complex
Elongation factor 2	ER	106	gi 119176	2; 2%	Promotes the GTP-dependent translocation of nascent protein chains from the ribosomal A-site to the P-site
Hypothetical protein LOC683313	?	96	gi 155369696	2; 3%	Intermediate filament protein
TMP21-1	Go	62	gi 3288599	1; 5%	Vesicular protein trafficking
Hormone/granin		998			
Secretogranin II	Gr	360	gi 38181552	8; 13%	Acidic soluble secretory protein
Chromogranin B	Gr	188	gi 2465398	7; 9%	Acidic soluble secretory protein
Chromogranin A	Gr	112	gi 127139019	4; 8%	Acidic soluble secretory protein
Neurosecretory protein VGF	Gr	99	gi 1352860	3; 7%	Involved in regulation of cell-cell interactions or in synaptogenesis
Chromogranin/secretogranin-like vesicle protein precursor	Gr	86	gi 111518	3; 6%	Involved in hormone sorting to secretory granule
Insulin 2	Gr	77	gi 9506817	2; 17%	Alters intermediary metabolism, stimulates glucose uptake

Table 14.1 (continued)

Protein	Location	Score ^a	NCBI accession number	[Peptides] ^{2+,3+} sequenced; sequence coverage	Function
Insulin 1	Gr	76	gi 9506815	2; 15%	Alters intermediary metabolism, stimulates glucose uptake
Protease					
Proprotein convertase subtilisin/kexin type 2	Gr	460 202	gi 6981342	4; 8%	Calcium-dependent enzyme responsible for tissue-specific processing of protein precursor molecules before and after di-basic residues
Carboxypeptidase E	Gr	195	gi 55249691	5; 14%	Involved in proteolysis and peptidolysis. Zinc carboxypeptidase. Implicated as a sorting receptor for regulated secretion
Cathepsin D	Ly	63	gi 115720	1; 2%	Limited specificity endopeptidase involved in intracellular protein degradation
Cell signalling					
GTP-binding protein alpha o	Mem	349 163	gi 8394152	4; 13%	G proteins are composed of three units: alpha, beta and gamma. The alpha chain contains the guanine nucleotide-binding site
GTP-binding G(olf) alpha subunit	Cy	91	gi 30387859	2; 7%	Mediates signal transduction within the olfactory neuroepithelium and the basal ganglia. May play role in visual transduction and mediate other hormones/neurotransmitters

Table 14.1 (continued)

Protein	Location	Score ^a	NCBI accession number	[Peptides] ^{2+,3+} sequenced; sequence coverage	Function
14-3-3 zeta isoform (protein kinase C inhibitor protein 1)	Cy	48	gi 52000883	1; 4%	Adapter protein that regulates a large spectrum of general and specialized signalling pathways. Binds to many partners, mostly by recognition to phosphoserine or phosphothreonine motifs
GTP-binding protein beta subunit 4	Cy	47	gi 62906844	1; 4%	The beta and gamma chains are required for the GTPase activity, for replacement of GDP by GTP and for G protein-effector interaction
Protein synthesis		214			
Ribosomal protein L18	Rib	95	gi 13592057	2; 13%	Protein synthesis
Statin-related protein or Eef1 alpha	Cy	71	gi 206440 and gi 1220484	2; 4%	Functions in the binding reaction of aminoacyl-tRNA (AA-tRNA) to ribosomes
Ribosomal protein L23A	Rib	48	gi 157818939	1; 8%	Binds to a specific region on the 26S rRNA
Metal binding		189			
Calreticulin	ER	123	gi 11693172	4; 8%	Calcium binding protein (also binds Zn). Also has chaperone activity interacting with PDIA3/ERp57 and with NR3C1
Calcium-binding protein p54/NEFA	Go	66	gi 14549433	2; 4%	Calcium-binding protein
DNA/RNA binding		125			
Heterogeneous nuclear ribonucleoprotein K	Nu	75	gi 16923998	2; 6%	Abundant, acidic (hnRNP) major pre-mRNA-binding protein. High affinity for poly(C) sequences

Table 14.1 (continued)

Protein	Location	Score ^a	NCBI accession number	[Peptides] ^{2+,3+} sequenced; sequence coverage	Function
Nucleophosmin	Nu	50	gi 7242160	1; 4%	Associated with nucleolar ribonucleoprotein structures. Binds single-stranded nucleic acids. Possible role in ribosomal assembly and transport
Na/K transport		105			
Na, K ATPase (alpha 1 subunit)	Mem	105	gi 205632	3; 3%	Sodium/potassium exchange; probably regulated by endocytosis

ER endoplasmic reticulum, Cy cytosol, Mt mitochondria, Mem membrane, CS cytoskeletal, Go Golgi, Gr granule, Ly lysosome
^aIndividual ions scores >46 indicate identity or extensive homology (*p*<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits

In order to focus exclusively on β -cell secretory granule-associated proteins, both groups employed several purification steps prior to protein identification analysis. These studies illustrate the principle that increased sensitivity and specificity can potentially be achieved by prior organellar purification.

Brunner et al. employed two sequential ultracentrifugation steps: a first-dimensional layered-discontinuous Nycodenz gradient followed by a Percoll cushion [22]. They monitored protein recovery by using an insulin immunoassay (granule marker) and western blotting for calreticulin and betagranin, respectively, as markers for ER and β -cell secretory granules. They then separated proteins by using one-dimensional SDS-PAGE and sectioning gels into 28 consecutive pieces, followed by in-gel tryptic digestion and protein identification analysis using LC-MS² followed by bioinformatic methods. They also monitored relative purity of granule preparations by western blotting for a series of markers associated with various extra-granular organelles. These findings pointed to the presence of lysosomes within their granule preparations, so the significance of the putative assignments of lysosomal proteins as components of the granule proteome remains to be confirmed.

By contrast, Hickey et al. chose to isolate β -cell secretory granules using a conservative methodology that initially employed two sequential orthogonal steps with concomitant monitoring for several, potentially confounding organelles including lysosomes, ER, mitochondria and cytosol [20]. The first of these used ultracentrifugation after overlaying INS-1E culture supernatants onto preformed, continuous OptiPrep gradients with subsequent fractionation and separation of granule-containing fractions, as determined by their hormone content [114], whose buoyant density was 1.10–1.11. They monitored this step using an insulin immunoassay as a granule marker coupled with multiple enzyme assays to track potential contamination with other organelles, including aryl sulphatase (lysosomal marker), NADH cytochrome C reductase (ER), citrate synthase (mitochondria) and lactate dehydrogenase (cytosol) (Fig. 14.7).

In the following, orthogonal step, they immunopurified granules by using anti-VAMP2 antibody-conjugated magnetic Dynal beads [20] and then undertook protein identification analysis that comprised the following sequential steps: reduction and S-carboxymethylation; snap-freezing and concentration by vacuum centrifugation; trypsinization; peptide recovery by batch reversed-phase chromatography; and LC-MS² with stringent bioinformatic criteria for inclusion in the final list.

14.4.3 How to Account for Contrasting Conclusions?

Although there are areas of substantive overlap in the results and conclusions, there were also broad areas of divergence between these two studies [20, 22].

Why might this be so? Both groups went to considerable lengths to ensure the purity of the granular preparations, whose contents they subsequently analysed. Hickey et al. assigned proteins to specific subcellular locations or functions by application of currently accepted rules. By contrast, Brunner et al. simply listed numerous mitochondrial proteins under the category of ‘other proteins’ (for example,

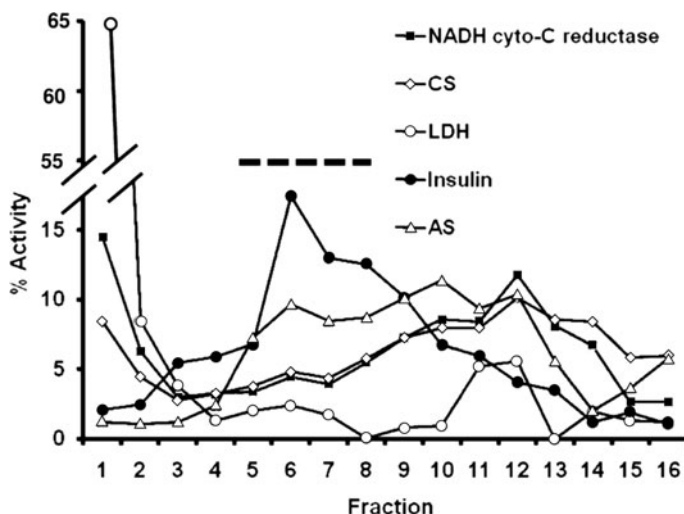


Fig. 14.7 Fractionation of lysates from cultured INS-1E β -cells was monitored by serial enzyme or hormone assays, as described [20]. Markers used were NADH cytochrome C reductase (endoplasmic reticulum), citrate synthase (mitochondria), lactate dehydrogenase (cytosol), insulin (granules) and aryl sulphatase (lysosomes). Insulin was concentrated within specific fractions (*broken line*). Insulin-containing fractions were then subjected to immunoaffinity purification using VAMP2. (Reproduced with permission from [20]). VAMP, vesicle-associated membrane protein.

10-kDa heat-shock protein; mitochondrial cystatin-C precursor; malate dehydrogenase, mitochondrial precursor; superoxide dismutase (whether this was SOD1 or SOD 2 they did not specify – there is evidence from others that both forms localize in mitochondria); voltage-dependent anion-selective channel protein 1 (VDAC); and ATP synthase α -chain, mitochondrial precursor), without allocating proteins to specific locations or tasks. Their findings with respect to this subgroup of proteins were generally consistent with those of Hickey et al, who also reported significant numbers of mitochondria-associated proteins in their granule preparations. Brunner et al. did not specify NCBI accession numbers for their individual proteins, so the exact molecular identity of some, for example, ‘superoxide dismutase’, remains uncertain.

Brunner et al. also reported an abundance of lysosomal proteins, which were annotated as hydrolases or lysosomal membrane proteins [22]. However, by contrast, none of these was detected by Hickey et al. [20].

14.4.4 Possible Explanations for Between-Study Divergence

Why were there such major differences between the two reports? Clearly, the two studies employed different isolation methods, so it was possible that there would be some differences between the sets of proteins identified. However, the divergences between large groups of proteins with shared prior subcellular localizations

indicate that more systematic factors were probably also at work. The lack of lysosome-associated proteins in the second study might reflect the use of enzyme markers during the fractionation which, when coupled with the following orthogonal immunopurification, permitted exclusion of lysosome-rich fractions from the final preparation.

In the reverse direction, 20% of the proteins identified by Hickey et al. were chaperones, whose main agreed functions reside in the ER, and a further 20% comprised other proteins with known ER- or Golgi-related functions (Fig. 14.8, Table 14.1). Clearly, the existence of a large number of chaperone-related proteins in the β -cell secretory granule, if confirmed, could have important implications for disorders triggered by protein misfolding, such as aggregation-associated β -cell degeneration [32].

However, the conclusions from these two preliminary forays into the field of β -cell secretory granule proteomics may best be viewed as providing the basis for further, intensive research.

The immunoaffinity purification procedures employed by Hickey et al. may have purified not only granules, but also aspects of the cytoskeletal apparatus responsible for granule transport, which therefore may adhere to them (for example, actin, tubulin and Rab GTP-binding proteins). Similarly, the presence of ER and cytosolic components in those results may follow from intracellular associations between secretory granules and aspects of the associated structures. As one example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was also

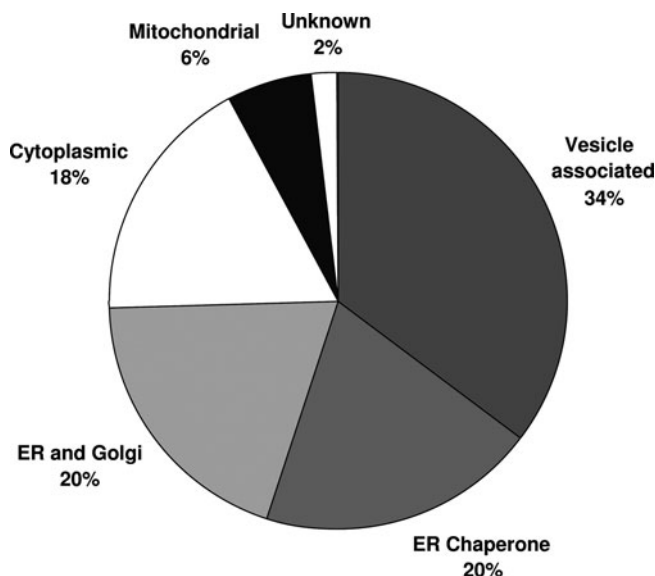


Fig. 14.8 Pie chart summarizing the cellular location of proteins identified in anti-VAMP2 antibody-conjugated magnetic Dynal bead-purified granule proteins, as listed in Table 14.1. (Reproduced with permission from [20]). *VAMP*, vesicle-associated membrane protein.

detected in that study, has several cellular roles and locations and is known to bind to microtubules [115].

However, there is evidence that attachments between secretory granules and components of the ER and cytoskeleton are physiological, so the findings of ER- and cytoskeleton-related proteins in purified granule preparations may well reflect these associations.

14.4.5 Calreticulin: Putative Assignment as an Insulin-Granule Protein or Indicator of ER-Protein Contamination?

Hickey et al. identified calreticulin in their β -cell secretory granule isolates [20], whereas Brunner et al. specifically excluded it from their ‘immature secretory granule (ISG)’ preparations [22]. The latter findings can be interpreted to indicate that the former preparations were contaminated with ER-derived proteins. Alternatively, calreticulin might be associated with a subset of granules (ISGs) not purified in Brunner et al.’s approach.

What does the balance of currently available evidence suggest concerning this interesting and potentially significant difference? There are reports that calreticulin fulfils distinct roles in different organelles. It has three functional domains (termed N, P and C). The first binds KXFFKR motifs, thereby acting as a chaperone [116], and also binds Zn^{2+} [117], which is noted to be present at high concentrations in the granule core. The latter two domains contain Ca^{2+} -binding sites and may thus function as local Ca^{2+} stores [118]. Although a COOH-terminal KDEL sequence could be seen as consistent with specific targeting to the ER, there is available evidence that calreticulin also localizes to cytoplasmic granules in T-lymphocytes [117] and sperm acrosomes and to the Golgi complex [119], and that it is systemically secreted to circulate in the plasma [120]. B-cell secretory granules contain a high- Ca^{2+} environment necessary for proteolytic processing [64, 73, 121, 122], so Hickey et al.’s findings could also be consistent with a role for calreticulin as a newly recognized Ca^{2+} storage protein in β -cell secretory granules.

The preceding analysis points to a series of specific and testable hypotheses. Evidently, an early consideration is that assignment of calreticulin as a putative insulin-granule protein requires independent confirmation.

14.4.6 How Might the Appearance of ‘Mitochondrial Proteins’ in ‘Insulin-Granule-Specific’ Preparations Be Interpreted?

The presence in insulin-granule preparations of proteins usually associated with mitochondria requires comment.

It is now becoming evident that apparently discrete organelles do not necessarily contain discrete protein sets. Interestingly, ‘organelle-specific’ proteomic analyses

have frequently demonstrated multiple locations for up to perhaps 40% of all component proteins [123]. Therefore, many proteins, which until now have not been associated with granules, may in fact be localized in or adherent to them and thus serve roles in granule function additional to those already assigned to them in other organelles.

For example, both studies [20, 22] detected component chains of the mitochondrial ATP synthase in purified granules, whereas until recently these subunits have mainly been considered to play roles in oxidative phosphorylation. However, the α -chain of ATP synthase was recently shown to act as a receptor for apolipoprotein A-1-mediated hepatic LDL endocytosis [124]. Thus, the current findings might signal that ATP synthase chains play further, hitherto unsuspected roles in β -cell secretory granule homeostasis.

A further explanation for the presence of a number of ‘mitochondrial proteins’ in β -cell insulin-granule preparations is that of the physical attachment between the two organelles that causes their obligatory co-purification. There is evidence that mitochondria are attached to cytoskeletal structures in hepatocytes, ciliary desmosomes [125], PC12 cells [126] and neurosecretory vesicles [127] and to pancreatic islet β -cell granules [62]. The proton-pumping requirements for granule maturation [128], within-granule chaperone activity and insulin exocytosis are dependent on mitochondria-derived ATP [126]. Furthermore, mutations in mitochondrial DNA can cause β -cell dysfunction and rare forms of T2DM [129]. The close proximity of mitochondria to granules could enhance efficient transfer of ATP to drive granule-associated processes, for example, their intracellular translocation, and within-granule protein processing, maturation and exocytosis.

14.4.7 Chaperones in the β -Cell Secretory Granule: Possible Implications

Aggregation of human amylin into β -sheet-containing oligomers has been linked to islet β -cell dysfunction and the causation of the common form of T2DM [32, 33, 93]. The existence of amylin misfolding in T2DM [108] points to a defect in regulation of the β -cell protein-folding pathways as a key target in the search for the underlying cause of islet β -cell dysfunction.

Within this context, the identification of specific chaperones in the β -cell granule provides a clear mechanistic linkage between protein misfolding in diabetes and a newly emergent, putative granule function. For example, alterations in the function of specific chaperones and their interactions with amylin can now be investigated as potential contributors to amylin misfolding. These findings represent a major potential contribution of proteomic analysis to the search for the molecular basis of disease mechanisms in diabetes.

In the study of Hickey et al., ER- and Golgi-associated proteins accounted for almost 40% of all those identified (Table 14.1), consistent with close association between β -cell secretory granules and elements of the ER and Golgi apparatus. Particularly abundant in these preparations were recognized chaperone proteins,

which comprised ~20% of all identified proteins in their preparations [20]. These data are consistent with the idea that β -cells may act as a significant source of secreted/circulating chaperone proteins. The localization of chaperones in β -cell granules might be considered unremarkable, given that they are abundant in most tissues and organelles. Consistent with these data, others have reported that HSPs and other chaperone proteins are plentiful in pancreatic islets [17, 130], where some are co-localized in secretory granules [56, 57, 131] or synaptophysin-containing microvesicles [132]. HSPs are also localized in the extracellular compartment [133] and their serum concentrations can vary according to physiological status [134].

Different chaperone molecules act cooperatively [134], consistent with the observed diversity in the β -cell secretory granules. HSP90 localizes in neuronal tissues [135], and a recent subcellular proteomic dissection of neuromelanin granules also reported high levels of ER-derived chaperones [136], consistent with its presence in pancreatic β -cells.

The apparent abundance of chaperones in β -cell secretory granules highlights their likely roles in preserving intact and well-regulated secretion of the hormones, amylin and insulin, and protecting the cell from the biological effects of protein misfolding [20, 32, 33]. The amylin-generated aggregates present in T2DM [27, 108] are similar to those that occur in Alzheimer's, Parkinson's and Huntington's diseases [137]. At high concentrations, dysregulated amylin folding occurs independently of protein convertases (PC2 or PC1/3), to potentially seed the amyloid fibrils that in turn form the tissue aggregates designated as 'islet amyloid' [81, 100, 138]. There is no direct evidence, however, for the presence of aberrantly processed human amylin in pancreatic islet amyloid [108]. Further proteomic analysis will be required to determine whether aberrant amylin processing is a contributor to islet amyloidosis and the origins of T2DM.

During its transition from soluble oligomers through protofibrils to insoluble fibrils [30, 31, 81, 100, 139], misfolding human amylin can elicit cytotoxic processes that can cause or result in β -cell death [83, 139]. This process in turn can cause decreased pancreatic β -cell mass and insulin secretory capacity, resulting in hyperglycaemia and, ultimately, T2DM [32, 33, 93, 140]. It may also serve as a target in the development of new classes of anti-diabetic compounds [32, 141].

14.5 Next Steps

This chapter has presented reasons for why it is desirable to undertake further detailed proteomic studies of the islet β -cell secretory granule. In particular, it is expected that important insights concerning the physiological regulation of islet hormone secretion and β -cell death, and the origins and mechanisms of T1DM and T2DM, might be obtained through such analysis.

However, the significant discrepancies between the two data sets discussed above [20, 22] show that currently available approaches have clear limitations. In particular, it is apparent that each new protein assignment to the islet β -cell granule

proteome must be regarded as no more than putative until substantive confirmatory evidence is obtained, by application of more selective proteomic analysis as well as one or more orthogonal methods, such as immunological co-localization of proteins to the granule. Results from even the latter must be viewed with caution, however, since limits of sensitivity and specificity of individual antibodies may provide limitations that must be taken into account.

At present, there are no available proteomic studies of β -cell granules from diabetic humans, so inferences concerning diabetes-related changes in granule composition and how such effects might contribute to the phenomena of diabetic β -cell degeneration must be drawn from other lines of investigation. There is a clear need for more precise information to characterize the changes in islet structure and function that occur in relation to the origins of β -cell dysfunction, islet amyloid formation and β -cell disappearance in T2DM.

It is to be hoped that the ongoing rapid development of new and improved proteomic approaches will enable such studies in the near future. Indeed, it is expected that one of the exciting outcomes of the current assembly of expert views in this volume will be the creation of linkages and methodologies by which these important goals can be facilitated.

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Chapter 15

Physiological and Pathophysiological Role of Islet Amyloid Polypeptide (IAPP, Amylin)

Gunilla T. Westermark

Abstract IAPP has a number of effects which may be of physiological relevance. Islet amyloid, which earlier was regarded as a non-important degenerative product, most likely plays a central role in the loss of β -cells in type 2 diabetes and probably in transplanted human islets. Taken together the results from human and animal studies show that amyloid develops before β -cell deficiency, and the occurrence of oligomers and amyloid intracellular induces β -cell death. Prevention of islet amyloid most likely will save β -cells and extend hormone secretion.

Keywords Islet amyloid · Islet amyloid polypeptide · IAPP · ProIAPP · ER-Stress · Apoptosis · Posttranslational processing

15.1 Islet Amyloid Polypeptide

Islet amyloid polypeptide (IAPP) was originally isolated as the major peptide constituent of the amyloid from an insulinoma [1] and subsequently isolated from amyloid deposits present in the islet of Langerhans from patients with type 2 diabetes [2, 3]. The 37 residue polypeptide proved to have an earlier unknown sequence, but showed an almost 50% identity to the known calcitonin gene-related peptide [4]. Other nomenclatures for IAPP are amylin [5], diabetes-associated peptide [6] and IAP (insulinoma amyloid peptide) [1]. IAPP is phylogenetically well preserved and found in all mammals where it has been looked for [7–10] and also in an avian [11] and fish [12].

During embryogenesis in mice, IAPP was detected in the primordia at E12 and the immunoreactivity was restricted to the simultaneously occurring insulin-expressing cells [13]. In human, IAPP immune-reactive cells were demonstrated

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from week 13 of gestation and here its expression was preceded by insulin that was present already at 9 weeks of gestation. In fetal and neonatal pancreas there were a higher number of insulin-positive cells than IAPP-positive cells, but this difference did not remain in the adult pancreas where all β -cells co-express insulin and IAPP [13, 14].

An additional expression pattern in developing mice was described by Wilson et al. [15] where IAPP and proglucagon/glucagon reactivity co-localized in the primordia at E10.5 in cells also expressing PC1/3, a convertase not present in the α -cells of the mature pancreas. Instead, PC1/3 is expressed in β -cells and together with proglucagon in intestinal L-cells. Cells positive for IAPP and glucagon did not express *pdx-1*, an activator of the IAPP gene [16], but they expressed brain-4 (*Brn-4*). *Brn-4*, originally described in brain, is also a regulator of glucagon expression in α -cells [17].

In human, IAPP is almost exclusively produced by the β -cell where it is stored [18] and released together with insulin [19] and only minor synthesis occurs in entero-chromaffine cells in the intestinal tract [20]. IAPP is synthesized as an 89 residue long prepropeptide [21, 22] from which an 18 residue signal peptide is removed in the endoplasmic reticulum. Posttranslational cleavages of proIAPP occur at di-basic residues and comprise the removal of N- and C-terminal flanking peptides. This proIAPP processing is initiated in the late trans-Golgi where cleavage by the proprotein convertase PC1/3 removes 16 residues at the carboxy terminus [23, 24] followed by PC2 cleavage in the secretory granules that leads to the removal of an 11 residue peptide at the amino terminus [25]. The residues Lys-Arg that remain at the C-terminus after PC1/3 cleavage are removed by carboxypeptidase E (CPE) [26]. To receive full biological activity, IAPP must be cyclized by a disulphide bond between the cystein residues at positions 2 and 7 of the mature IAPP and be C-terminally amidated [27]. An additional processing site is present at residues 79–80 (Lys-Arg) of the C-terminal flanking peptide, but no extended IAPP peptide has been described (Fig. 15.1).

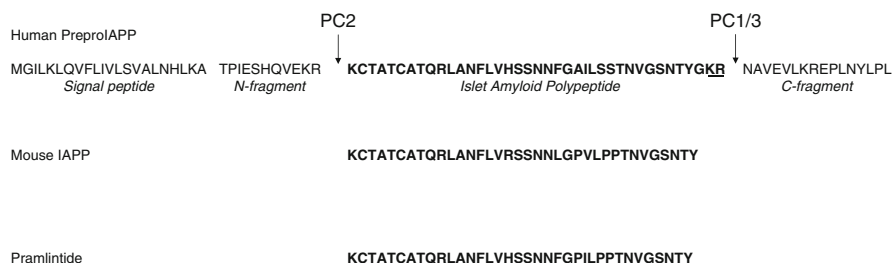


Fig. 15.1 The one-letter code of the human preproIAPP sequence. The cleavage positions for signal peptide and the N- and C-terminal fragments are marked by spaces. Prohormone convertases PC2 and PC1/3 cleave proIAPP at the N- and C-terminus, respectively. Processing sites are marked by arrows. The C-terminal underlined residues KR (lysine-arginine) will be removed by carboxypeptidase and the C-terminal G (glycine) is used for C-terminal amidation. The sequence for mouse and rat IAPP. The sequence for pramlintide with the three proline substitutions underlined.

Proinsulin is processed to insulin by the same convertases at the same location [28], and IAPP and insulin are stored in the same secretory granules [29–31]. In the mature granule IAPP and C-peptide occupy the halo region while Zn^{2+} insulin is present in the dense core region [32]. The insulin-to-IAPP ratio varies but is often reported to be 10:1 [32–34]. However, heterogeneity among β -cells occurs [35]. Reported non-stimulated plasma levels of IAPP in man range between 2 and 20 pM IAPP [36, 37]. IAPP is cleared by the kidneys [38] and insulin is cleared by the liver and kidneys [39, 40]. The clearance of IAPP is almost four times slower than that determined for insulin but comparable to that for C-peptide [41]. Taking this into account, a comparison of IAPP and C-peptide plasma levels might be more accurate and reflect the actual ratio.

The IAPP-to-insulin ratio remains constant under normal circumstances and is not affected by the type of stimuli [42]. However, assays used for IAPP quantification will not discriminate between the active hormone and the partially or non-processed hormone.

15.2 Regulation of the IAPP Gene

The human IAPP gene is a single-copy gene situated on the short arm of chromosome 12 and consists of three exons separated by a 0.3 and 5 kb intron, respectively. Exon 1 encodes most of the 5' untranslated region of the transcribed RNA while exon 2 encodes the signal peptide and 5 residues of the N-terminal flanking peptide and exon 3 encodes the remaining residues 6–89 of the preproIAPP molecule [21, 43–46]. Transcription of the IAPP gene is controlled by a promoter situated within the sequence spanning from –2798 to + 450, relative to the transcriptional start codon [47]. IAPP and insulin genes contain similar promoter elements [48] and the transcription factor PDX1 regulates the effects of glucose on both genes [49–52]. Glucose-stimulated β -cells respond with a parallel expression pattern of IAPP and insulin [53, 54]. The islet hormones interplay in the regulation of glucose homeostasis [55], and insulin and glucagon stimulate IAPP gene expression [16], in contrast to somatostatin that has no effect [56].

15.3 Receptor for IAPP

IAPP belongs to the calcitonin family of peptides also including calcitonin [57], calcitonin gene-related peptides (CGRP) [58], adrenomedullin [59] and intermedin [60]. For long, there was a futile search for a specific IAPP receptor, and it was not until the discovery of the receptor activity modifying proteins (RAMPs) the problem was solved. RAMPs constitute a family of three different single transmembrane proteins [61] that by combining with the G-protein-coupled calcitonin receptor (CTR) or the calcitonin receptor-like receptor (CLR) [62] determine the ligand specificity

and also increase the receptor repertoire [63]. Pairing of RAMP3 with a CT receptor forms an IAPP-specific receptor [64].

15.4 IAPP in Other Species

A more disperse distribution of IAPP is seen in other species. In rat and mouse, IAPP immunoreactivity co-localizes partly with gastrin, somatostatin and peptide YY in enteroendocrine cells in the gastrointestinal tract [65, 66]. In pancreas IAPP is present in β - and delta-cells [67]. IAPP is expressed in the rat brain and sometimes with a different distribution of that shown for CGRP [68].

In chicken the IAPP immunoreactivity was co-localized with insulin in the small islets, but mRNA expression analysis revealed higher signals from intestines and brain [11]. In fish, IAPP immunoreactivity is present in the islet organ Brochmann body [12] and in the intestinal tract.

15.5 Physiology of IAPP

15.5.1 Glucose Regulation

Taken together the results from a large group of researchers suggest that IAPP exerts an autocrine or paracrine effect on β -cells and acts as a modulator of insulin secretion [69–71]. Glucose-stimulated insulin secretion from perfused rat pancreas can be inhibited by IAPP at 75 pmol/l, a concentration equal to that determined in the effluent from rat pancreas. The inhibitory effect on insulin secretion is limited to physiological changes of glucose and no effect remains when glucose levels are augmented from 5.5 to 16.6 mmol/l [72]. Insulin secretion in response to other secretagogues such as sulphonylurea that block ATP-dependent K channels or KCl that depolarized β -cells is also markedly reduced by IAPP [73]. IAPP infusions in rats with hyperglycaemia clamped at 11 mmol/l showed a dose-dependent reduction in insulin secretion, and 8.5 and 85 pmol/min reduced plasma insulin by 31 and 53%, respectively [74]. The inhibitory effect on insulin secretion ceased by time and IAPP is suggested to be a short-time regulator of insulin secretion [74]. Immunoneutralization of intra-islet IAPP by specific antibodies or by the IAPP inhibitor IAPP 8–37 potentiates both glucose- and arginine-stimulated insulin release [71]. This is in accordance with the finding that IAPP null mice have a more rapid glucose clearance in response to both oral and intravenous administered glucose [75]. This phenotype was reversed by the introduction of human IAPP in the IAPP null mice.

15.5.2 Peripheral Effects of IAPP

There are great differences in the reported in vitro and in vivo effects on IAPP and some of these differences could be ascribed to the use of pharmacological levels of IAPP, the solubility of IAPP or other not yet known circumstances.

An early reported effect for IAPP was the reduction of basal and insulin-stimulated glycogen synthesis in rat skeletal muscle [76]. In the work by Furnsinn et al., short-term IAPP infusion reduced glycogen content in the hindlimb rat muscle [74]. Again, this effect was only seen after short-time IAPP exposure and did not remain after long-term exposure. IAPP can regulate glycogen synthesis by activation of glycogen phosphorylase and inactivation of glycogen synthase [77–79], effects antagonized by IAPP 8–37 [79]. IAPP has also been shown to cause peripheral insulin resistance in vivo in cat [80], rat [81] and dogs [82]. In contrary, Kassir et al. failed to measure any change in the insulin-stimulated glucose disposal rate in dog [83].

A single injection of IAPP was shown to partly inhibit glucagon release in freely fed mice while IAPP had no effect after a glucose load. Glucagon secretion stimulated by L-arginine was reduced by IAPP while glucagon stimulated by hypoglycaemia was unaffected [84]. In cat, rat-IAPP injection 5 min prior to intravenous administration of arginine or glucose lowered plasma glucagon levels and reduced also the insulin levels [85]. Therefore, one effect for IAPP secreted together with insulin in response to a rise in blood glucose may be to modulate postprandial glucagon secretion.

15.5.3 Gastric Emptying

Administration of IAPP has been shown to delay gastric emptying and thereby reduce the increase in postprandial glucose [86–88]. Due to the absence of endogenous IAPP in type 1 diabetes gastric emptying is expected to be accelerated. When this was monitored in 21 patients with type 1 diabetes mellitus, no significant difference in mean or median time compared to the controls could be detected. However, it should be pointed out that a large variation occurred among the individuals with type 1 diabetes and increased emptying occurred in a sub-group without secondary complication [89]. However, in a recent study by Heptulla et al., it was hypothesized that accelerated gastric emptying should occur in children with complication-naïve type 1 diabetes. Instead they found delayed gastric emptying when compared to controls [90]. Therefore, administration of IAPP in individuals lacking the hormone seems to have a different effect than expected on gastric mobility.

15.5.4 Regulation of Food Intake

The central high affinity binding sites for IAPP are concentrated to nucleus accumbens, area postrema and in the immediate adjacent nucleus of the solitary tract in rat [91] and monkey [92]. Centrally [93–96] and peripherally [97–99] administered IAPP reduces food intake and produces anorexia in mouse and rat. Chronic subcutaneous infusion of IAPP, at concentrations kept within the pathophysiological range, causes a dose-dependent reduction of food intake and body weight gain by lowering the adiposity [99]. The anorectic effect from chronic peripheral infusion

was abolished in rats after AP/NST lesion [100], and the effects of intraperitoneal injections of IAPP were reduced by direct injections of IAPP receptor antagonist AC187 into the area postrema [95]. Chronic intraperitoneal infusions of AC187 increased the total food intake in genetically obese fa/fa rat but were ineffective on lean littermates.

IAPP can enter the blood–brain barrier [101] and the anorectic effects exerted by peripheral IAPP indicate that the molecule might be a satiety factor. One side effect found during the clinical trials with the IAPP analogue pramlintide was a slight weight reduction in the study group.

The expression pattern of intracranial IAPP is somewhat unclear and local expression was reported to occur at multiple sites in the rat brain [68, 102]. Whether IAPP produced at this site participates in the regulation of food intake has yet to be resolved.

In a study on postpartum mRNA regulation a 25-fold increase of IAPP was detected in the preoptic area of the hypothalamus. The increase was verified at the peptide level and suggests that IAPP plays part in maternal regulation [103].

15.5.5 Calcium Metabolism

IAPP participates in the regulation of total bone mass and stimulates osteoblast proliferation and bone formation, in both rodent and human [104, 105] cultured osteoblasts. Bone absorption is reduced because IAPP slows down the mobility of the osteoclasts [106–108] and prevents the fusion of the preosteoclasts into multinucleated osteoclasts shown in rodent cell culture [109]. IAPP null mice have a 50% reduction in bone mass when compared to wild-type mice [110]. It still needs to be elucidated if IAPP has any significance on the development of osteopenia, but IAPP fasting levels are reported to be significantly lower in patients with osteoporosis and in women with anorexia nervosa, a disease frequently associated with osteoporosis [111].

15.5.6 IAPP as a Drug in Obesity and Diabetes Treatment

Pramlintide/symlin is a synthetic analogue of IAPP with three structure-breaking proline substitutions inserted at positions 25, 28 and 29 to inhibit aggregation of the peptide (Fig. 15.1). This exchange of residues makes pramlintide more like the rat IAPP. Symlin was approved by US Food and Drug Administration (FDA) in 2005 [112], but not elsewhere, to be given together with insulin to control post-meal blood glucose in patients with type 1 and type 2 diabetes. Over the recent years, multiple clinical trials on the effects of pramlintide have been undertaken. Overall, the reported biological effects include an often mild weight decrease, significant reduction in HbA1c and a decrease in insulin dose. The drawback was the experience of a transient nausea.

More interesting is the finding from ongoing clinical studies where a combination of pramlintide and the leptin analogue metreleptin was given and resulted in an approximately 13% weight loss after 24 weeks, a reduction significantly more than after treatment with pramlintide or metreleptin alone [113].

15.6 Amyloid

15.6.1 Amyloid in General

Amyloidoses constitute the largest group among the protein misfolding diseases, and today, 30 different proteins have been characterized from amyloid deposits in human [114]. The proteins are unrelated and each protein is linked to a specific amyloid disease. Based on the distribution pattern, the diseases are divided into two forms: systemic amyloidosis where deposits are present throughout the body and where the precursor proteins most often are plasma proteins; and localized amyloidosis where the deposits mainly are restricted to the site of production and where not all but many of the precursors are polypeptide hormones.

Amyloid

Amyloid designates a proteinous aggregate with specific fibrillar appearance. Hitherto, 30 different proteins have been described to form amyloid in humans, and each protein is associated with a specific disease. *Islet Amyloid PolyPeptide (IAPP)* is a β -cell product that under normal condition participates in the regulation of blood glucose, but aggregates and forms amyloid in the islets of Langerhans in the majority of individuals with type 2 diabetes. The formation of amyloid fibrils involves the formation of smaller intermediates, the so-called oligomers, which have been ascribed the cell toxic activity and are believed to cause the β -cell reduction present in conjunction with type 2 diabetes. Also, amyloid aggregates have a potential to form barriers that destroy the islet architecture and interfere with cell signalling.

Further Reading:

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For the association of amyloid to other diseases see, e.g., John H (2009), The amyloid hypothesis for Alzheimer's disease: a critical reappraisal. *J Neurochem* 110(4): 1129–1134

Added by the editors

Amyloid is often referred to as an amorphous material, but it consists of fibrillar structures with a diameter of 7–10 nm and of indefinite length. The protein molecules that make up the fibrils are aligned perpendicular to the fibrillar axis and

this is believed to cause the specific tinctorial characteristic of amyloid, affinity for Congo red and green birefringence in cross-polarized light.

Formation of amyloid can be separated into three separate phases: a lag phase, an elongation phase and a plateau phase. The lag phase is of undefined length and can last from minutes up to a lifetime. It is during this period the monomer unfolds and forms small amyloid aggregates. These aggregates can act as templates, and amyloid fibrils extend from these during the elongation phase. The elongation of fibrils will continue until the plateau phase is reached, dependent on the equilibrium for the specific peptide [115, 116]. In experimental in vivo and in vitro models for amyloid formation, the introduction of a minute amount of preformed amyloid fibrils can dramatically shorten the lag phase and cause rapid amyloid formation. It is evident that extracellular amyloid can be degraded and cleared, but often the formation of amyloid exceeds the resolution and therefore the amyloid mass will continue to grow as long as the precursor is supplied.

15.6.2 Islet Amyloid

Islet amyloidosis is a localized form of amyloid disease and was described by Opie in 1901 [117]. Islet amyloid is the main islet pathology present in individuals diagnosed with type 2 diabetes, but the reported frequency of amyloid varies from 40 to 100% [118–122]. This rather large discrepancy in amyloid frequency between reports and the presence of amyloid in islets of non-diabetic subjects have questioned the importance of islet amyloid as a cause of type 2 diabetes. In a study by Maloy et al., amyloid was present in 59% of the subjects with diabetes but when the group was subdivided depending on treatment, it was shown that the patients that received insulin treatment all had islet amyloid [119]. This points to an association between the severity of the disease and the prevalence of islet amyloid.

In addition to man, islet amyloid occurs in primates [123–125] and cats [126]. In primates, there is an increased risk for spontaneous diabetes when kept in captivity and the disease development includes obesity and hyperinsulinaemia, a disease pattern that resembles the type 2 diabetes that develops in humans. There are three studies on different monkeys that connect islet amyloid with the development of diabetes. In *Macaca nigra*, the amyloid area was determined in pancreas biopsies and on autopsy in 18 monkeys, some followed for 10 years [127]. The amyloid area was determined and compared to the result of an intravenous glucose tolerance test. In non-diabetic monkeys the amyloid area did not exceed 3% and no abnormalities in insulin secretion or glucose clearance were detected. When the amyloid load progressed and affected 20–40% of the islet area both insulin secretion and glucose clearance were decreased. Diabetes shown by hyperglycaemia developed when the amyloid area exceeded 50–60%. In *Macaca mulatta* the progression of the metabolic deterioration was correlated to the islet morphology present in autopsy biopsies [125]. Animals were divided into four different groups: (1) lean

young monkeys, (2) monkeys >10 years old, (3) monkeys with normoglycaemia and hyperinsulinaemia and (4) diabetic animals. In group 3 the β -cell volume was increased while group 4 animals had a reduced β -cell volume. Amyloid deposits were present to a varying degree in 4 of 6 group 3 animals replacing 0.03–45% of the islet mass. In the diabetic group amyloid was present in 8 of 8 animals and the affected area varied between 37 and 81% of total islet area. In the third study performed on 150 baboons, the metabolic state was correlated to the islet amyloid mass and the result thereof showed that the levels of fasting plasma glucose were sensitive and specific enough to determine the extent of amyloid [123]. The latter is different from studies on human where islet amyloid was significantly associated with a higher mean HbA_{1c} but not with fasting blood glucose levels [128].

Islet amyloid does not develop in mouse or rat. This depends on the amino acid composition, and especially the three proline substitutions present at positions 25, 28 and 29 in rodent IAPP are assumed to prevent amyloid aggregation [129]. In the model for human IAPP fibril formation presented by Jaikaran et al. the regions made up by residues 1–17, 18–27 and 30–37 form strands that fold and form intramolecular beta-sheet structures while the residues at positions 17–19 and 28 and 29 form the beta-turns. The presence of proline residues, which are known as beta-strand breakers, at positions 28 and 29 will disrupt the structure and prevent fibril formation (Fig. 15.1) [130].

CD analysis of human IAPP in monomeric form revealed mainly random coil structure [131, 132], and NMR analysis on human IAPP and rat IAPP when bound to membrane showed alpha-helical content in the N-terminus [133].

The presence of amyloid in the islets of Langerhans in the South American rodent *Octodon degu* was surprising since the predicted IAPP sequence after cDNA analysis from degu revealed a non-amyloidogenic IAPP sequence with protective proline residues at positions 28 and 29 [134]. Interestingly, an insulin sequence was obtained when the degu islet amyloid was sequenced [135]. Degu insulin sequence diverges from human and rat insulin at 32 out of 53 positions [134] and these differences could result in a potentiated amyloidogeneity. The degu develops diabetes when kept in captivity, and therefore, despite the different origin of the amyloid in the islets of Langerhans in degu it points clearly to the importance of amyloid in the islets.

15.6.3 Amyloid in Transgenic Animal Models

The original data on islet amyloid derive from studies performed on material recovered post-mortem and we are still waiting for new methodology that will allow in vivo studies on islet amyloid in humans. Meanwhile studies have been performed on transgenic animals which have been very useful and facilitated a large number of studies on IAPP cell toxicity and amyloid formation and allowed the exploration of the role of different pathways in amyloidogenesis.

Several transgenic mouse strains that express the human IAPP gene linked to rat insulin I or II promoter [136, 137], cDNA for human IAPP linked to the rat insulin II promoter [138] or cDNA for human IAPP linked to the human insulin promoter have been established. Also a transgenic rat strain expressing the cDNA encoding human IAPP driven by the rat insulin II promoter (HIT rat) has been constructed [139]. A strain that expresses human IAPP, but made deficient for endogenous IAPP expression was made by crossing a transgenic mouse with an IAPP-deficient strain. Expression of the human IAPP gene in the IAPP null mice ameliorated the defect insulin secretion detected in this strain. Formation of amyloid caused solely by over-expression of human IAPP was only found in one mouse strain [140]. In other strains, amyloid occurred in mice fed a diet high fat [141, 142] after treatment with dexamethasone or growth hormone [143] or when introduced into a diabetogenic trait [144]. In human IAPP transgenic ob/ob mice the extensive IAPP production caused amyloid to form in parallel with the development of insulin deficiency and persisting hyperglycaemia [144]. In the HIP rat, over-expression of human IAPP led to spontaneous development of hyperglycaemia in transgenic rats by the age of 4 months and overt diabetes was present in all rats by the age of 10 months. In these animals the amyloid amount did not correlate to the fasting blood glucose. Instead a positive relationship between β -cell apoptosis and fasting blood glucose was reported.

An earlier prerequisite in the definition of amyloid was that it should be present extracellularly and this was also the main finding in the post-mortem material, often affected with massive amyloid load and autolysis. However, some amyloid present in insulinoma [145] and human islets transplanted to mice [146] appeared to be present intracellularly. In transgenic mice or in cultured islets isolated from such animals, it was shown that initial amyloid formation occurs intracellularly [140, 142, 147]. The amount of amyloid deposited in cultured islets was clearly dependent on the glucose concentration.

15.6.4 Oligomers and Cell Toxicity

In some amyloid diseases it has been clear that the massive amyloid burden does not always correlate to the clinical picture. Instead, the attention was drawn to the fibril formation process and it was shown that aggregation to amyloid fibrils involves formation of intermediates, and these oligomeric assemblies are ascribed to the cell toxic effect. The term oligomer is still a matter of debate. It does not define a homogenous population of aggregates and the number of monomers varies.

Most of the results on oligomers arise from studies on A- β , the amyloid protein deposited in the Alzheimer brain where soluble oligomers have been implicated as the toxic species, responsible for cell death [148, 149]. When Lorenzo et al. added mature IAPP fibrils to β -cells in culture they detected apoptosis. With today's knowledge, it is most likely that oligomers were present in the solution and the propagation of amyloid fibrils induced apoptosis [150]. The general mechanism is

supported by the existence of antibodies that recognize cell toxic oligomers independent of the nature of amyloid protein [151]. Different models of how oligomers exert their toxic activity exist. An early finding [150] was that A-beta can form ion-leaking channels in lipid layers [152, 153]. Human IAPP was also shown to form active channel structures while this was not seen by rat IAPP [154]. Atomic force microscopy studies on channel structures suggest that the IAPP channel consists of five IAPP molecules [155]. A second model for IAPP toxicity is membrane permeabilization during fibril elongation [156, 157]. The N-terminal part of human and rat IAPP contains alpha-helical structures and can interact with the membrane, but only human IAPP can aggregate and form the amyloid fibrils that disrupt the membrane. The result of this model fits well with the electron microscopical picture on amyloid interaction with β -cells (Fig. 15.2).

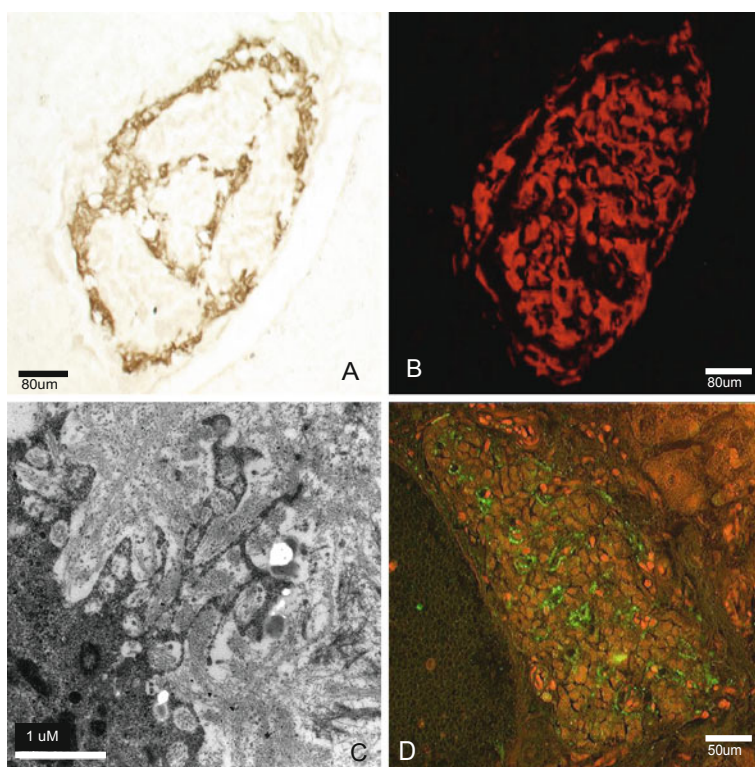


Fig. 15.2 (a) Islet of Langerhans from a patient with type 2 diabetes immunolabelled with antibodies against insulin (*brown*). (b) Consecutive section stained for amyloid with Congo *red*. The section is viewed in fluorescence microscope at 540 nm. (c) Electron microscopical section of human islet with amyloid. Note the close association between the bundles of amyloid fibrils to the β -cell membrane. (d) An isolated islet transplanted to the liver. Cell nuclei are *red* and amyloid *green* (from [182]).

Being a secretory protein, IAPP will after synthesis enter the secretory pathway starting with the endoplasmic reticulum where the SS-bond is formed and eventual further folding is assisted by chaperons, transported to Golgi and finally to the secretory granule where the main part of the posttranslational processing occurs. The mature proteins are stored in the secretory granules, waiting for secretion. If not used the granule content will be degraded by crinophagy.

Type 2 diabetes is often preceded by peripheral insulin resistance that is compensated for by an increased insulin biosynthesis. This increase in the demand on the secretory machinery in the β -cells can cause endoplasmic reticulum (ER)-stress which can induce apoptosis if not compensated by activation of the unfolding protein response (UPR). The UPR response includes upregulation of ER-resided chaperones to assist folding of aggregated proteins, a selective inhibition of protein synthesis to reduce ER workload in favour of synthesis of proteins that augment UPR and transport of misfolded proteins to the ubiquitin-proteasome system (UPS) for degradation. It has been shown that over-expression of IAPP in cell lines and in the HIP rat activates apoptosis and reduces the β -cell number [139, 158]. A sixfold increase of CHOP positive islet cell nuclei was detected in sections from patients with type 2 diabetes. No reactivity was detected in sections from non-obese or obese non-diabetic subjects [159]. The stress-inducible transcription factor CHOP is present in the ER and if activated during ER-stress, it will translocate to the cell nucleus. An increased production of the ER-stress markers HSPA5, CHOP, DNAJC3 and BCL2-associated X protein was detected in human pancreatic islets recovered from diabetic subjects [160]. However, in this immunological study CHOP reactivity appeared to be restricted to the cytosol without translocation to the cell nucleus.

The association between human IAPP expression and ER-stress induction is still contradictory. Hull et al. failed to detect changes in the mRNA expression of the ER-stress markers Bip, Atf4 and CHOP and splicing of Xbp1 mRNA in mouse islets expressing human IAPP after culture in 11.1, 16.7 and 33.3 mmol/l glucose [161]. The islet amyloid that developed was associated with reduced β -cell area in a glucose- and time-dependent manner. In a recent paper from Peter Butlers research team, where the commercially available oligomer antibody A11 was used, oligomers were found intracellularly in human islets from patients with type 2 diabetes [162]. The oligomers disrupted the membranes of the secretory pathway and entered the cytosol. Oligomers were also found in close association to mitochondria.

15.6.5 IAPP in the Secretory Granules

A fibrillar material recognized by proIAPP specific antibodies is present in the halo region of the secretory granules in β -cells affected by small amounts of amyloid. When the intracellular amyloid mass expands, granule-sized aggregates fuse and replace the cytosol. Cells stained for intracellular amyloid are also recognized by

the apoptosis marker M-30 [164]. During the hyperinsulinaemic period that precedes diabetes there is an increase in secretion of proinsulin and partially processed proinsulin (32–33 split proinsulin) [165, 166]. Because proIAPP is processed by the same convertases a similar change of processing of proIAPP is expected with an increase in secretion of IAPP bound to the N-terminal propeptide (N-IAPP). When human β -cells were incubated in 20 mmol/l glucose the cellular content of insulin was decreased without a concomitant decrease of IAPP resulting in a shift in IAPP-to-insulin ratio. Western blot analysis of cell content showed a raise in proIAPP and an intermediate that in size corresponded to N-IAPP [167]. Expression of human proIAPP in B-TC 6 cells that express PC2 and PC1/3 and where proIAPP is expected to be processed into IAPP failed to show amyloid formation. Expression of proIAPP in GH4C1 cells that lack PC2 and PC1/3 or AtT-20 cells that lack PC1/3 and where aberrant processing of proIAPP occurs lead to amyloid formation [168].

IAPP is known to be one of the most amyloidogenic peptides and is readily assembled into amyloid fibrils, and the absence of fibrillar aggregates in the granules during non-pathological condition raises the question of whether an endogenous inhibitor is present in the secretory granule. It was shown that IAPP aggregation was in a concentration-dependent manner inhibited by insulin [32, 169]. Therefore, a change in the intragranular milieu may be enough to facilitate aggregation of proIAPP/IAPP. When the composition of endocrine granules was determined it was shown that chaperones were present. This shows that assisted folding may be of importance also at this site [170].

It is possible that two different ways exist for IAPP to reduce the β -cell number in islets of Langerhans in patients with type 2 diabetes. One is through formation of oligomers that induce ER-stress ultimately leading to apoptosis. Amyloid formation has been suggested to primarily constitute a surviving pathway where formation of fibrils is a way to neutralize toxic oligomers. However, intracellular growth of amyloid which replaces the cytoplasm may also induce apoptosis.

15.6.6 Mutations in the IAPP Gene and Amyloid

Mutations in the IAPP gene occur both in the coding region and in the regulatory part of the gene. The most studied mutation is the S20G, present in the Asian population [171]. In a search for mutations within the coding region of IAPP, 294 patients with type 2 diabetes were analysed and the S20G mutation was found in 4.1%, but was absent in the control group and in patients with type 1 diabetes. In a more comprehensive study that included >1500 Japanese subjects with type 2 diabetes the mutation was found in 2.6% and it was concluded that IAPPS20G is linked to an increased risk for the development of this disease [172]. A study performed on a Chinese population identified the mutation in 2.6% of the individuals with early-onset type 2 diabetes but in none of the control subjects. Screening for the mutation in other populations failed to identify the S20G variant [173]. There is an increase in the fibrillation propensity of S20G IAPP in vitro [174, 175] and expression of the

mutant in Cos-1 cells induced more apoptosis [175]. The in vitro findings indicate that S20G may form more cell toxic amyloid in vivo. A gene promoter polymorphism in the region -132 G/A of IAPP has been identified in a Spanish population. The frequency of the G/A genotype was 9.7% in the studied 186 individuals with type 2 diabetes and 1.5% in the non-diabetic control group [176]. The presence of the mutation has been shown to increase the basal transcriptional rate of the IAPP promoter [177]. This is interesting for the amyloidogenesis since over-expression and increase of the amyloid precursors is believed to trigger amyloid formation. However, the search for the promoter mutations in other countries has failed to show association to type 2 diabetes or islet amyloid load [178].

Genome-wide associated (GWA) studies performed in Caucasian [179, 180] and in Han Chinese [181] populations have until now identified 20 different polymorphisms with shifting associations to type 2 diabetes (T2D), and hitherto, neither of the pinpointed loci include IAPP.

15.6.7 Importance of Amyloid in Transplanted Islets

Impact of amyloid in transplanted human islets is a fairly new field. Islet transplantation as a possible strategy to restore or improve the glucose homeostasis in patients with type 1 diabetes was tried out already in the 1970s, but with low success [182]. Despite major changes in, e.g., islet isolation protocols, transplantation procedure and immune suppression regime few recipients remained insulin independent 1 year after transplantation. Over the years many experimental transplant studies have been performed with rat and mouse islets which are protected against islet amyloid formation (see above). In a study from 1995, human islets were implanted under the kidney capsule of nude mice which were either normoglycaemic or made diabetic with alloxan [146]. The implants were recovered after 2 weeks and, surprisingly, amyloid was detected in 16 out of 22 transplants (73%) after Congo red staining or by immune electron microscopy. There was no difference between diabetic and non-diabetic recipients. Further studies on transplanted human islets showed that amyloid formation was not restricted to kidney implants and amyloid developed to the same degree in human islets implanted to the spleen or liver [183].

Experimental studies with transgenic mouse islets, expressing human IAPP, have verified the findings. A graft containing 100 islets isolated from transgenic mice were implanted under the kidney capsule on mice with streptozotocin-induced diabetes. The graft was sufficient for adjusting the blood glucose level, but over the 6 following weeks an increase in plasma glucose concentration was detected but was not seen in mice transplanted with non-transgenic mouse islets. The implants were recovered after 6 weeks and amyloid was found in 92% of the transplants with transgenic islets and the β -cell volume was reduced by 30% [184].

Studies in human material have of natural reasons been very limited. We have, however, studied the amyloid content in human islets implanted to the liver of a type 1 diabetic man, dying from a myocardial infarction [185]. The recipient received

three different grafts and was off insulin treatment for a period between transplantations. Amyloid was found in about 50% of the islets identified in the liver. This finding clearly points to amyloid as an important factor for loss of graft survival. Is it possible to extend the survival of transplanted islets? Marzban et al. reduced the proIAPP expression by 75% through the introduction of short interference (si) RNA in human islets kept in culture [186]. The reduction of proIAPP synthesis reduced the amyloid load by 63% in islets cultured for 10 days. The results indicate that the proIAPP synthesis most likely must be abolished if amyloid formation should be prevented.

15.7 Conclusion

Taken together the results from the animal studies show that amyloid develops before β -cell deficiency, and the occurrence of oligomers and amyloid intracellular induces β -cell death. Prevention of islet amyloid will save β -cells and extend hormone secretion.

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Part IV
Physiological, Pharmaceutical and Clinical
Applications and Perspectives

Chapter 16

Present State of Islet Transplantation for Type 1 Diabetes Patients

Torbjörn Lundgren and Olle Korsgren

Abstract Islet transplantation can today be offered to well-selected patients with type 1 diabetes who have previously received a renal transplant or have severe problems with hypoglycaemic unawareness. Even if patients need repeated transplantations to become insulin independent, a stabilization of glucose levels and normalization of HbA1c are often achieved already after the first transplantation. In this chapter we describe the historical background to today's transplantations and in higher detail discuss the findings of clinical trials performed in recent years, starting with the "Edmonton Protocol". Practical issues surrounding islet transplantation and available methods to monitor the islet graft's performance are discussed in separate sections.

Keywords Islet transplantation · Clinical · Outcomes

16.1 The Prospects of β -Cell Replacement Therapy in Type 1 Diabetes

Over the last 50 years transplantation has emerged as the treatment of choice for a wide range of diseases. Today thousands of kidneys, livers, hearts, lungs and pancreases are transplanted at an increasing number of transplant centres worldwide each year. Often the transplantations serve to replace an organ where many of its functions and morphological integrity have been lost (i.e. liver cirrhosis or polycystic kidney disease). However, there are several examples where only a part of an organ's repertoire of functions is distorted. Such are when liver transplantation is performed with the sole purpose to enable the patient to produce a hormone or

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enzyme [1, 2] or when the whole bone marrow is replaced because of one malignant cell line, failure to synthesize haemoglobin or for various immunodeficiencies [3].

In type 1 diabetes (T1DM) the failure of one type of cell causes a systemic disease that without replacing the hormone, i.e. insulin, leads to death. Even when treated with insulin, but without the minute to minute feedback system normally present within the lost β -cells, T1DM in the long run may lead to blindness, amputations, renal failure and premature death. It is today widely accepted that the angiopathy leading to these complications is a result of the inferior metabolic control of today's insulin administration regimes in comparison with that in a non-diabetic person. Taking this into account, β -cell replacement therapy through transplantation could play an important role. The provision of a sufficient β -cell mass holds a promise to fine-tune blood glucose through the production and release of hormones in a physiological manner, restoring normoglycaemia and avoiding long-term complications.

The main hurdle in the advancement of transplantation in general has been how to evade the allogenic barrier of the immune system. In any transplantation between two individuals immunosuppressant drugs are needed to avoid rejection of the transplant (see Section 16.3). Another hurdle to take into account when replacing the destroyed cells in the treatment of T1DM is the autoimmune process involved in the aetiology of the disease.

The β -cells of a human are spread within the islets of Langerhans which themselves are spread diffusely within the pancreas. The total volume of the islets is about 1–2 ml and represents only 1–2% of the pancreas tissue. When considering β -cell replacement therapy there are today two options: either to replace the whole organ, pancreas transplantation, or to prior to transplantation separate the islets of Langerhans from the exocrine tissue, islet transplantation.

Islet transplantation has several theoretical advantages compared to whole-organ transplantation. It is a minimal invasive treatment, islets can be pretreated to avoid rejection or to enhance and document performance. In the future islets could potentially be derived from stem cells securing β -cell availability.

16.2 The History of β -Cell Replacement Therapy

The first series of *pancreas transplantations* was performed by Lillihei, Kelly and co-workers at the University of Minnesota in the 1960s [4]. Today more than 23,000 pancreas transplantations have been reported to the International Pancreas Transplant Registry [5]. Advancements in organ procurement, surgical technique and immunosuppressive medication have improved results over time. The most common technique today places the new pancreas in the abdomen with arterial vascular supply coming from the recipient's iliac artery. The recipient's own pancreas is left in place. Venous drainage of the graft can lead to either the portal vein or the systemic system. Commonly a piece of the donor's duodenum comes with the

graft and is anastomosed to the recipient's jejunum to drain the pancreatic exocrine secretions.

Combined *pancreas/kidney transplantation* is a widely accepted therapy for T1DM patients with end-stage renal disease. Three years after transplantation 84.7% of the patients are insulin independent with functioning kidney grafts [6]. Corresponding results with pancreas transplantation alone or transplantation performed after kidney transplantation with grafts from different donors are 74.9 and 78.0%, respectively.

Pancreas transplantation is considered to be a major surgical procedure, and short-term complications involve cardiac morbidity, pancreatitis and intra-abdominal infections.

Ironically, the complications of pancreas transplantation emanates from the exocrine portion that only serves as a carrier for the endocrine tissue. Minimal invasive methods to transplant isolated islets have been developed in parallel with the clinical advancements of pancreas transplantation. Already in 1972 Lacy and co-workers [7] could cure chemically induced (alloxan, streptozotocin) diabetes in rats through intra-abdominal or intraportal transplantation of isolated islets. To isolate large numbers of islets from human pancreases proved to be more difficult. The inability to transplant enough viable islets hampered the possibilities to perform clinical trials. In 1988 Ricordi introduced an islet isolation technique that improved outcomes of human islet isolation [8], and in 1990, the St. Louis group reported the first insulin-independent type 1 diabetic recipient transplanted with islets from two deceased donors [9]. Exogenous insulin, however, had to be reinstituted on day 25 after transplantation, probably due to rejection. Warnock and colleagues published the first case with a patient remaining insulin independent more than a year after transplantation in 1992 [10] after receiving islets from a total of five donors equalling about 10,000 islet equivalents (IEQ) per kilogram body weight. Results were slowly improving but clearly inferior to those of pancreas transplantation. Still at the end of the last millennium insulin independence rates at 1 year were about 10–15% after islet transplantation (ITR Giessen, www.med.uni-giessen.de/itr).

16.3 Immunosuppression

In any transplantation between individuals (allotransplantation) it is necessary to give immunosuppressive medication to avoid *rejection*. The only exception to this rule is transplantation between identical twins. When transplanting islets to treat T1DM, also the underlying autoimmunity of the disease must be considered. If not, diabetes can reoccur in the transplanted tissue [11]. The importance of *autoantibody* titres is controversial. There are reports indicating inferior results in pretransplant autoantibody-positive patients, but others have seen no such correlation [12, 13]. However, in most cases the same immunosuppression used to avoid alloimmunity seems to keep autoimmunity at bay.

The immunosuppressive drugs used can be divided into induction therapy that is given for a short period in conjunction to the transplant and maintenance therapy that the patient has to use during the lifetime of the graft.

For maintenance, combinations of drugs are given. This is both to limit the side effects of high doses of any specific drug and to benefit from the different mechanisms of action between the drugs. Introduction of the first calcineurin inhibitor, cyclosporine A, in the late 1970s helped to improve short-term results in all types of organ transplantation. Triple treatment with cyclosporine A, azathioprine (a proliferation inhibitor) and steroids became the main elements of all transplantation protocols for many years. In the last 10 years tacrolimus (a second-generation calcineurin inhibitor) to a large extent has replaced cyclosporine A and mycophenolate has replaced azathioprine.

Sirolimus was introduced in 2000. This was the first drug in a new category (mTOR inhibitors) also targeting IL-2, but at a different level and considered less nephrotoxic than both cyclosporine A and tacrolimus. Hopes were high that sirolimus would be able to replace the calcineurin inhibitors. This has, unfortunately, not been fulfilled. Sirolimus is today used in combination with other drugs and has been commonly used in islet transplantation protocols (Section 16.5).

The pancreas is considered more prone to rejection than other organs, and induction therapy with lymphocyte-depleting antibodies is common. In islet transplantation the trend is currently shifting from IL-2 receptor blockers (daclizumab and basiliximab) that were used in the original Edmonton Protocol [14] to depleting antibodies (anti-thymocyte globulin) (www.citregistry.org).

Some specific *side effects of immunosuppressive drugs* are found in Tables 16.1 and 16.2. The therapy as such carries important general side effects that are not

Table 16.1 Common drugs in maintenance therapy

Generic name	Brand names	Introduced	Mechanism of action	Specific side effects
Prednisolone	Prednisone®	1950	Anti-inflammatory	Osteoporosis, insulin resistancy
Azathioprine	Imuran® Imurel®	1960	Limits expansion of white blood cells	Neutropaenia
Cyclosporine A	Sandimmune® Neoral®	1979	Inhibits T cell proliferation Calcineurin inhibitor	Nephrotoxic, hypertension
Tacrolimus	Prograf® Advagraf®	1995	Inhibits T cell proliferation Calcineurin inhibitor	Nephrotoxic, diabetes
Mycophenolate	CellCept® Myfortic®	1995 2004	Limits expansion of white blood cells (lymphocytes)	Upper GI symptoms, neutropaenia.
Sirolimus	Rapamune®	2000	Inhibits T cell proliferation by blocking intracellular signalling	Impairs wound healing, mouth ulcers, interstitial alveolitis, hyperlipidaemia
Everolimus	Certican®	2003		

Table 16.2 Common drugs in induction therapy and rejection treatment

Generic name	Brand names	Introduced	Mechanism of action	Specific side effects
Methyl prednisolone	Solu-Medrol®	1950	Anti-inflammatory	Osteoporosis, insulin resistancy
Daclizumab	Zenapax®	1999	IL-2 receptor blockers	
Basiliximab	Simulect®	1999		
Anti-thymocyte globuline	ATG-Fresenius®	1999	Depletes lymphocytes	Allergic reactions, neutropaenia
	Thymoglobuline®	2002		

specific for any drugs, but rather the level of total immunosuppression. This involves all types of infections (bacterial, viral and fungal) and is most common early after transplantation when the total levels of the drugs are high. Through the years clinicians have become more acquainted with the present therapies, and generally the accepted trough levels of the calcineurin inhibitors have been lowered. Severe infections are less common today than 10 or 15 years ago. Routine antibacterial and antiviral prophylaxis is given for the most common and dangerous microbes during the first months after transplantation. The immunosuppressive agent dosages are tapered to lower levels during the same time period but must be taken during the lifetime of the graft.

There is an elevated frequency of cancer in transplanted patients. The most common are skin cancers as basalioma and squamous cell cancer, but the accumulated risk for cancer can be calculated to be four times to that of the general population [15].

16.4 Indications for Clinical Islet Transplantation

As mentioned above immunosuppressive therapy is, at least today, compulsory for clinical islet transplantation. This medication constitutes the largest risk for the patient, since the islet transplantation procedure as such is considered safe if patients are screened for bleeding abnormalities, etc. [16]. However, even in the most successful cases exogenous insulin is traded for immunosuppressive drugs. This limits the indications of the procedure since most patients with T1DM function well on conventional therapy.

There are three generally accepted situations where islet transplantation can be considered.

- The patient has already undergone a transplantation of another organ (usually kidney) and is therefore already on immunosuppressive medication. Here the patient can benefit from the islet transplantation without having to balance it with the added risk of starting immunosuppressive treatment. This category is most often referred to as “islet after kidney” (IAK).

- The patient has end-stage renal disease and is planned for transplantation with a kidney from a diseased donor. Here one can add islets processed from the same donor, *simultaneous islet kidney transplantation* (SIK). Often islets from one or two additional donors are transplanted to potentiate the graft. The most common indication for pancreas transplantation is *simultaneous pancreas kidney transplantation* (SPK). The procedure is, however, limited to relatively young and physically fit patients receiving a pancreas from a young and non-obese donor. Islet transplantation usually comes into question for patients who do not qualify for SPK or at centres/regions/countries that do not perform SPK.
- *Islet transplantation alone* (IA) has been the most common procedure during this century (www.citregistry.org). The main indication is frequent hypoglycaemia combined with an unawareness of symptoms of low blood sugar. Iatrogenic hypoglycaemia is a major unresolved problem for many patients with T1D. It is the limiting factor in the management of T1D, causing some deaths as well as recurrent physical, and recurrent, or even persistent psychosocial morbidity [17].

16.5 Results Obtained in Clinical Islet Transplantation Trials 2000–2009

Coming into the new millennium insulin independence through islet transplantation was rare. During the last years of the decade 10–15% of the treated patients were insulin independent at 1-year post-transplant; however, up to 80% still had function shown by measurable C-peptide (ITR Giessen, www.med.uni-giessen.de/itr).

Clinical trials in islet transplantation have generally been small. There are no published prospective randomized clinical trials to date. The collaborative islet transplantation registry (CITR) (www.citregistry.org) has collected data from 412 allo islet recipients transplanted from 1999 to 2008 at 27 North American, 3 European and 2 Australian centres and publishes annual reports.

In July 2000 the group in Edmonton, Canada, reported [14] that they had achieved insulin independence in seven consecutive cases with a follow-up of 4–15 months. Several things had been changed compared to previous protocols. First – all patients received islets due to severe problems with hypoglycaemia (islet alone, IA). Second – a new steroid-free immunosuppressive regime was used with daclizumab as induction therapy and sirolimus plus tacrolimus as maintenance. Third – transplantations were repeated with islets from several donors [2–4] until the patient became insulin independent. Fourth – islets were transplanted fresh. No period of culture preceded the transplantation. A mean of $11,547 \pm 1,604$ islet equivalents per kilogram body weight (IEQ) was needed for the recipient to obtain insulin independence.

A study was initiated by the Immune Tolerance Network spreading the “Edmonton Protocol” to nine centres on both sides of the Atlantic. Each centre transplanted —three to five patients. Here the results were more complex with varying results between centres. At one centre all four transplanted patients were insulin

independent at 1 year and thereby fulfilling the primary endpoint. On the other hand there were three centres with a total of 11 transplanted patients where none reached the primary endpoint. In total 44% were insulin independent at 1 year and 58% had been so at least at one time point within the trial [18]. A larger series of transplantations conducted in Edmonton showed that while the patients still had islet function at 5 years (showed as C-peptide production in >80% of the patients) and an absence of hypoglycaemia the median time until exogenous insulin was reinstated was 15 months and only 7.5% were insulin independent at 5 years [19]. Similar results are presented in the CITR report (www.citregistry.org). Of the registered patients 70% were considered insulin independent (at least 14 days without insulin) but only 39% remained so after 2 years. Thirty-five per cent had lost all function 3 years after the last transplantation.

In 2004 the Minneapolis group presented a trial with eight patients all becoming insulin independent after only one transplantation and one donor (mean $7,271 \pm 1,035$ IEQ/kg) [20]. Key features in the protocol included aggressive anticoagulant therapy surrounding the transplant, etanercept (a TNF- α inhibitor) and rabbit anti-thymocyte globulin induction. Five out of eight remained insulin independent after 1 year. There was a large discrepancy between the donors (101 kg BW/34 BMI) and the recipients (60 kg BW/23 BMI), making the results of the single donor procedure difficult to interpret. The same group have later reported an insulin independence of 66% with a mean follow-up of 3.4 years [21]. This would indicate an improvement compared to the corresponding Edmonton figures of about 25% at 3 years. However, the study includes only six patients and is too small to draw any firm conclusions.

Rickels et al. published a series of reports on the metabolic evaluation and performance of patients transplanted with islets (IA) [22, 23], clearly indicating an insufficient β -cell mass (22% of normal) even in insulin-independent persons, suggesting that only a fraction of intraportally transplanted islets actually engraft in the liver (Section 16.7).

Further follow-up on the Edmonton patients has shown a reduction of GFR and progression of albuminuria over 4 years of observation despite improved glycaemia [24]. The rate of decline in GFR was, however, extremely variable and difficult to predict. Tacrolimus or the combination of tacrolimus/sirolimus was primarily thought to be responsible for the findings; however, further progression of diabetic nephropathy could not be ruled out. In a crossover study in 42 patients by Warnock et al. [25], there was on the contrary found to be no reduction of renal function after islet transplant compared to the general public or the group that received intensive medical therapy. The same study showed a reduction of 0.9% in HbA1c and less progression of retinopathy in the islet-transplanted group.

The researchers in Edmonton have also published data showing that 31% of their recipients had developed de novo *HLA antibodies* after transplantation [26]. Twenty-three per cent were still on immunosuppressive medication when the first antibodies occurred. In the patients who had discontinued this medication for various reasons (predominantly graft failure) 10/14 were broadly sensitized with a mean PRA of 89.5%. These antibodies will make it more difficult to find a suitable donor if the patient would be considered for a pancreas, islet or kidney graft in

the future. Cardani et al. [27] confirmed that all of their patients who had stopped immunosuppression had developed *alloantibodies*. Considering development of HLA antibodies while on immunosuppression Ferrari-Lacaraz and colleagues did not find that they were more frequent than in kidney transplantation [28, 29].

The studies above and others have in the last decade showed that insulin independence can be obtained by multiple islet infusions and in some cases by only one. Even in these successful cases the engrafted β -cell mass is only about a fourth of that of a healthy individual. Considering that the cut-off level where insulin has to be reinstated is in the region of 20%, only a slight reduction in β -cell mass must occur before this line is crossed and the patient has to start taking insulin again. However, the most common indication for islet transplantation during this decade has been recurrent severe hypoglycaemia. To overcome these problems independence of exogenous insulin is desirable but not needed. A lower degree of function from the islets is usually enough [19]. In fact, more than 80% of the patients in the Edmonton series enjoy freedom from recurrent hypoglycaemia for more than 5 years but only 40% of the total in the CITR registry.

At its present state islet transplantation should be seen as a procedure in development, suitable for carefully selected and informed patients. The ITN trial showed the importance of experience and devotion at all levels. It can be easily argued that islet transplantation should only be performed at centres with a special interest in β -cell replacement therapy and as often as possible within clinical trials. Prospective randomized trials are needed to firmly establish which patients benefit from the procedure and how to perform it most efficiently.

16.6 Practical Issues in Clinical Islet Transplantation Today

The pancreas from the diseased donor is harvested and transported the same way regardless if the pancreas is intended for islet isolation or whole-organ transplantation. Acceptable donation criteria differ from centre to centre concerning age, weight, previous illnesses, current medication, etc. In the Nordic countries we accept all donated pancreases for isolation that come from non-diabetic donors where the kidneys are deemed suitable for renal transplantation. Since pancreas transplantation is an established procedure with superior clinical results, whole-pancreas transplantation has priority if the pancreas is thought suitable for a specific patient on the waiting list.

Islet isolation is a demanding, labour intensive and expensive procedure, more so than the transplantation itself and follow-up of the patients. This has motivated the Nordic transplantation departments (Uppsala, Stockholm, Malmö, Göteborg, Oslo and Helsinki) and others [30–32] to set up networks with a central islet isolation facility serving more than one islet transplantation unit. Pancreases are sent to the isolation lab for processing, and islets are returned for transplantation. This also allows for easier exchange of islets suitable for specific patients across the network. However, most international islet transplantation groups still have their own isolation facility.

As in all other transplantations the so-called *cold ischemia time* (CIT), the time between when blood supply is stopped in the donor and later is reinstated in the recipient, is critical with inferior results if it is prolonged. In islet transplantation it is the start of the isolation and not reinstated circulation that is set as the endpoint for CIT. Attempts have been made in recent years to introduce new and innovative transport solutions to reduce the impact of transportation and CIT on islet isolation and transplantation outcome and thereby also allowing longer CIT. Despite promising early results in animal models, results in large clinical trials have been disappointing [33, 34].

Islet isolation can be divided into four steps.

1. The duodenum, excessive fat and connective tissue are removed from the graft and the pancreatic duct is located.
2. Collagenase, responsible for disintegrating the tissue, is infused into the pancreatic duct.
3. The pancreas is put into a chamber together with the collagenase at a specific temperature. In addition to the enzymatic process the chamber is shaken to physically separate the tissue. This incubation is stopped when samples taken from the chamber show free islets.
4. The tissue fragments are separated from each other in a cell centrifuge through the use of the different densities between the exo- and endocrine tissue.

Collagenase quality is of critical importance to the result of the isolation and may, unfortunately, vary between both producers and individual batches. This is one obstacle hindering the production of large amounts of high-quality islets from every isolation. If optimal donor and transport criteria are fulfilled, an experienced islet isolation facility succeeds in producing islets suitable for clinical transplantation in ~50% of the isolations [18]. It can be calculated that up to 80% of the original pancreases β -cell mass is then successfully retrieved [35]. Islets vary in size from only a few cells to half a millimetre in diameter. To standardize quantification of the graft a standard islet equivalent (IEQ), with a diameter of 0.15 mm, has been used. Islet transplantation protocols often call for 5,000 IEQ per kilogram body weight of the recipient or more to be transplanted.

Quality tests that are performed on the islet graft include viability tests, glucose stimulation tests and screening for microbes.

Following a successful isolation, islets can either be transplanted “fresh”, as in the classical Edmonton Protocol or be kept in culture awaiting transplantation. Clinical outcomes using the two approaches do not differ, and today most groups prefer the later which has some substantial practical advantages. The time in culture allows for quality tests to be performed on the isolated islets, cross-matches made against possible recipients and finally travel to the transplant centre and pre-transplant preparation for the chosen patient. The transplantation hereby becomes a planned and most often daytime procedure. Islet transplantation is normally performed within 72 h after islet isolation.

Access to the portal vein for islet transplantation into the liver is established either through a percutaneous transhepatic puncture guided by ultrasound or through a small abdominal incision with cannulation of a mesenteric vein. Different centres have different access to angiography suites/interventional radiologists and operating theatres/surgeons. Using the percutaneous route the portal vein can be reached through a ventral puncture aiming at the left portal branch or lateral puncture aiming at the right branch. In our experience it is quicker and easier to get a good central placement of the catheter tip if the later technique is used. A problem with the lateral approach is, however, that breathing movements may dislocate the catheter.

Islet transplantation using the transhepatic technique can be performed using local anaesthesia often combined with sedation. The patient is kept euglycaemic through administration of i.v. insulin. All modern clinical protocols administer heparin (500–7,000 U) with the islet graft to avoid clotting and thrombosis. This gives a certain risk for bleeding from the surface of the liver at the puncture site when the catheter is removed. This risk can be lowered by leaving a foam plug [16] at the exit site or delay the removal of the catheter allowing coagulation parameters to normalize and local deposits of fibrin to counteract bleeding.

Islets have been shown to be vulnerable to glucotoxicity [36]. Clinical results improve when patients have been kept euglycaemic in the peri- and post-transplant period [20]. Often tapering of insulin is not started until engraftment is expected to be finished after about a month. This is in sharp contrast to pancreas transplantation which usually normalizes blood sugar levels already at the reestablishment of blood circulation.

Most immunosuppressive protocols in islet transplantation contain diabetogenic drugs, primarily tacrolimus (www.citregistry.org). Balancing between efficacy and negative side effects calls for frequent contact with the patient and monitoring of the patients general condition, glucose levels and immunosuppressive drug levels. In the Nordic Network for Clinical Islet Transplantation the patient daily reports the last 24-h pre/postprandial and bedtime glucose levels together with insulin doses and hypoglycaemia (if any) through telephone, fax or e-mail to the transplant centre during the first month. After discharge from the hospital, trough levels of immunosuppression are measured twice a week together with C-peptide, creatinine and other lab tests. If no complications occur, outpatient visits are kept once a week for the first 3 months following the transplant. Thereafter, visits and blood draws are spaced out in steps to once every 3 months. Decisions whether to repeat the transplantation are discussed and made together with the patient. Stabilization of blood sugar and normalization of HbA1c are most often achievable already after one transplant. If this was the goal for the transplantation and since we know that even when insulin independence is achieved it is usually lost within 2–5 years, it could be argued that a specific patient would benefit to accept a certain dose of exogenous insulin and only repeat islet transplantation when or if the initial positive result show signs of deterioration. In our experience, patients who have had severe problems with fluctuating blood sugars and hypoglycaemia often cherish an improvement in this regard.

The History of Transplantation

Throughout history people have always been intrigued by the possibilities of the transplantation of organs and tissues from one body to another. During the fifteenth century we can find references in historical medical literature of attempted blood transfusions as well as the transplantation of teeth (presumably from cadavers). A skin transplant and a corneal transplant were reported in medical journals dating as far back as 1880. Of course, these early attempts at transplantation were usually unsuccessful. It was not until early in the twentieth century that transplantation offered the promise of renewed health and life envisioned by our ancestors.

Some highlights in transplantation history:

- 1905 First corneal transplant by the ophthalmologist Eduard Zirm.
- 1908 First skin allograft.
- 1908 Successful first cadaver knee joint transplant.
- 1918 First blood transfusion.
- 1954 First successful living-related kidney transplant from identical twins performed in Boston, MA. The recipient had normal kidney function for 8 years.
- 1963 First liver transplant performed.
- 1963 First lung transplant performed at the University of Mississippi Medical Center, Jackson, MS.
- 1967 First heart transplant performed by Dr. Christian Bernard at Groote Schuur Hospital, South Africa. The recipient had normal heart function for 19 months.
- 1967 First successful pancreas transplant performed by Dr. Richard C. Lillehei at the University of Minnesota, MN.
- 1968 Brain death criteria created.
- 1979 Living related pancreas transplanted, Minneapolis, MN.
- 1981 First heart and lung transplant performed at Stanford University Medical Center, Stanford, CA.
- 1984 First heart/liver transplant performed at the Children's Hospital of Pittsburgh, PA.
- 1984 Baby Fae receives a walnut-sized baboon heart in an operation at Loma Linda University Medical Center, CA. She was the first infant to receive an animal organ. Baby Fae lived for 21 days.
- 1989 First liver transplant from a living related donor.
- 1999 First successful pancreatic islet transplantation following optimized immuno-suppression scheme, the Edmonton Protocol.

Adapted from: <http://www.thetransplantnetwork.com> and <http://www.organtransplants.org>.

Added by the Editors

16.7 The Liver as the “Gold Standard” for Clinical Islet Transplantation and Alternative Sites

Islet infusion into the liver via the portal vein has been the “gold standard” for clinical islet transplantation. This is a quite different procedure compared to other

transplantations where blood supply is secured by anastomosing the transplanted tissues blood vessels surgically to the recipient's corresponding arteries and veins. In animal models several different transplantation strategies (i.e. deposition under the kidney capsule or intrasplenic) have functioned well, but in non-human primates and humans intraportal islet transplantation is the only technique that routinely has led to insulin independence. The efficacy is, however, low and little is known for certainty about how the engraftment procedure takes place in humans. Until engraftment is fully established, which can take months [37], islets have to rely on oxygen and nutrition to be delivered by the portal blood flow. This makes them vulnerable to a series of unwanted influences during this period, i.e. high levels of immunosuppressive drugs in the portal vein, hyperglycaemia, IBMIR (see below) and low oxygen content. In humans, islets do not seem to become incorporated in the liver parenchyma as in rodents, but rather stay in the portal vein lumen or wall [35]. Since transplanted islets do react to glucose in a proper way in humans already shortly after transplantation [14] and studies in rodents show that islets transplanted intraportally into the liver are exclusively stimulated to insulin release through the hepatic artery [38], this also shows that transplantation in rodents is a poor model to understand engraftment in human allo transplantation.

Islet transplantation into the portal vein elicits a reaction called the instant blood-mediated inflammatory reaction (IBMIR) [39, 40]. *IBMIR* is induced by tissue factor (a transmembrane glycoprotein expressed on islets, i.e. within vessel walls) and is a non-specific reaction activating both inflammatory and coagulation pathways. It has been shown to be detrimental to the islet graft with up to 50% of islets lost already at the transplantation [41]. Tissue factor is downregulated during islet culture, and this is another reason that most groups today have abandoned the original Edmonton Protocol to transplant islets immediately following islet isolation. Clinical trials trying to abrogate IBMIR through the selective blocking of coagulation and complement are presently ongoing.

The above-demonstrated disadvantages concerning intraportal islet transplantation have stimulated investigations aiming to find sites better suited to harbour the graft. Striated muscle [42], omental pouch [43] and the native pancreas [44] are among the suggested sites, but all have yet to show equal or better efficiency than the intraportal route in humans.

16.8 Monitoring the Islet Graft

Clinical studies show that islet function is lost over time (Section 16.4). Many theories have been presented on why this occurs (i.e. rejection, toxicity of drugs and exhaustion because of too little islet mass from the beginning). Tools to monitor the islet graft are of vital importance to understand why, when and how function is decreasing. It would also allow treatment to be started if an unwanted development occurs, i.e. rejection. However, methods to do this efficiently have been lacking. Metabolic evaluations have been adapted from endocrine research,

e.g. insulin requirements, p-glucose, p-C-peptide, HbA1c, continuous subcutaneous glucose monitoring (CGMS) and stimulation tests, i.e. mixed meal tolerance test (MMTT), IV glucose tolerance test (IVGTT) and glucose-potentiating arginine stimulation test. Some of these can be applied to calculate the functional β -cell mass, but it is usually too late to salvage function that has been lost between measurements, and it gives no indication as to why the function has been influenced. More specific calculations for islet transplantation have been constructed (Beta Score [45], CGGCR/CPGR [46], SUI index [47]), but they invariably rely on the factors related to above and have no further predictive value. Islets can be found in needle biopsies from the liver, but only in about 30% of the cases and even when they are found they do not reflect overall graft function [48]. *Islet imaging* is one of the fields where progress has been made in recent years. Ideally one should be able to detect the islets and to get a three-dimensional description to see where changes have occurred and where ongoing inflammation/rejection is to be found. This could allow stereotactic-directed biopsy and subsequent microscopic analysis. Imaging should also allow quantification of the functioning β -cell mass and consistently enable comparison both between individuals and between different time points in the same patient.

Clinically applied approaches involve *positron emission tomography* (PET) and *magnetic resonance* (MR). In both instances islets have been labelled in vitro, transplanted and subsequently followed by imaging in the recipient. This limits the interval when the graft can be monitored since the label will be cleared from the graft. Ideally one should be able to inject an islet or β -cell-specific probe and that way allow repeated imaging of the graft. To find such a probe has proved more difficult than originally thought [49].

¹⁸Fluorodeoxyglucose has been used to label the islets to allow *PET-CT imaging* during the peritransplant period. ¹⁸Fluorodeoxyglucose is the most commonly used PET tracer having a half-life of 109 min, allowing investigators to follow the labelled islets for about the same time period after transplantation [41, 50]. The primary findings were that islets were unevenly distributed in the liver with large portions of the graft caught in “hot spots”, presumably clots, and that less than 75% of the infused islets could be found in the liver at the end of the transplantation. This substantial loss of islets during the actual transplantation procedure is due to an injurious innate immune reaction (IBMIR) [39] (Section 16.7).

When using MR, the islets have been labelled with superparamagnetic iron oxide particles [51]. Islets have been followed as dark signal voids in post-transplant liver images up to 6 months. However, no correlation was found when compared with the number of transplanted islets or clinical islet graft function. The total number of spots was generally low (maximum 138), and it can be assumed to reflect the same phenomena, with many islets engrafting together, that in the PET studies was referred to as “hot spots”.

A complicating feature is that transplantation usually has to be repeated for the patient to become insulin independent. This makes it difficult to evaluate each islet dose and to identify critical parameters in the donors, islet isolation processes, cultures, transports or transplantations and post-transplantation patient management

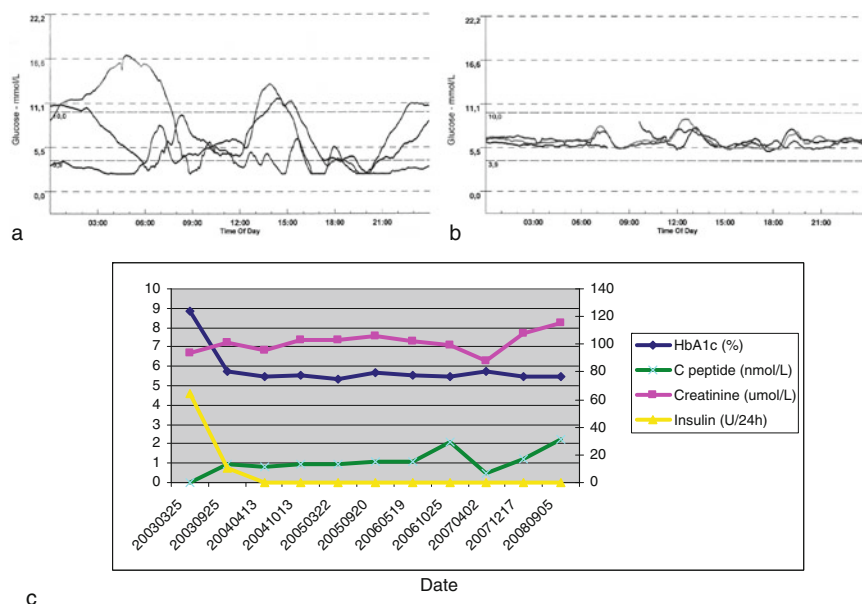


Fig. 16.1 Continuous subcutaneous glucose monitoring (CGMS) and outcome in clinical islet transplantation. (a) Typical accumulated 72 h CGMS in a patient screened for islet transplantation, (b) CGMS at the 5-year follow-up visit in a patient within the Nordic Network for Clinical Islet Transplantation who had previously received a kidney (IAK) and (c) Clinical parameters in the same patient as in (b). He was transplanted with four doses of islets from March to August 2003.

that are contributing to the final outcome. In spite of these shortcomings there has been a gradual and continuing progress in clinical outcome after islet transplantation (Fig. 16.1).

When islet transplantation can be performed with a 1:1 ratio between donors and recipients, giving long-lasting insulin independence and low acceptable side effects of the immunosuppression, availability of islet tissue will become a prime issue. T1DM is a much more common disease than end-stage organ failure of heart, liver or kidneys, where demand exceeds availability of organs today. Development in stem cell research is driven in parallel with allo islet transplantation, and hopefully the first clinical trials can be started within the new decade.

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Chapter 17

Predictive Protein Networks and Identification of Druggable Targets in the β -Cell

Joachim Størling and Regine Bergholdt

Abstract A prerequisite for designing good drugs that perform through clinical development with the final goal to treat human diseases is a detailed understanding of the mechanisms underlying disease. This is particularly true for complex diseases such as diabetes. It has become increasingly clear that complex traits or phenotypes are the result of an interplay between environmental factors and numerous genes and proteins that jointly affect the functionality of biological systems. Since interactions between proteins in networks and pathways make up biological systems, it is essential that we learn more about how networks and pathways are influenced by environmental factors and genetic variation and how such influences cause disease. In this chapter, we will discuss recent data, advancement and ideas on how more valid druggable targets to treat diabetes may be predicted by the application of bioinformatics and systems biology.

Keywords β -cells · Diabetes aetiology · Drug targets · GWAS · Phenotype description · Protein networks · Systems biology

17.1 The Need for New Ways of Identifying Druggable Targets

Tens of billions of Euros and dollars are spent each year by the pharmaceutical industry on the development of new drugs to treat human diseases. However, drug discovery is an extremely expensive and risky business, and despite the enormous investment in drug discovery, the rate of failure of drug candidates in clinical development is dreadfully high. One explanation is that the strongly restricted genetic and epigenetic backgrounds and environmental settings of simple animal and in vitro cell systems used to model human disease and preclinical drug testing differ greatly from the genetically, environmentally and epigenetically much more

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heterogeneous nature of the human population. Another explanation is that drug discovery traditionally has been aiming at designing drugs against targets considered to affect simple biological systems or signalling pathways, and such an approach represents an exceedingly simplistic view of the mechanisms underlying complex human diseases [1]. An improvement of the success rate of drugs in clinical development will require new approaches to pinpoint more valid drug target candidates for pre-clinical testing. Obviously, a prerequisite for this will be an improved understanding of disease mechanisms and increased insight into the complex biological systems in tissues and cells in a heterogeneous human population. This is the true challenge and entails innovative ways of studying disease and disease model systems and highlights the need for systems biology and bioinformatics approaches.

Drug Development

Drug development defines the entire process of bringing a new drug to the market. The process implies the identification of drug targets, drug synthesis, characterization, screening and assays for therapeutic efficacy. When a drug has proven valuable in these tests, it will enter the process of drug development prior to clinical testing. Although great advances in technology and insight into biological systems, drug discovery remains a lengthy, difficult, expensive and inefficient process.

Recent studies have estimated the cost of developing a new drug to be between USD 500 million to 2,000 million. Hence, there is a huge demand by the industry to reduce cost of drug development, which may be obtained by more careful drug target selection by the integration of novel techniques and disciplines such as bioinformatics and systems biology.

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17.2 How Can Drug Target Identification Be Optimized?

Improved prediction of valid drug targets will require increased insight into the specific biological and molecular systems in tissues and cells that are responsible for causing disease. Most human diseases, including type 1 and 2 diabetes which are the result of complete or relative destruction and dysfunction of the β -cells, are caused by a complex interplay between environment and genes. The interaction between environmental factors and the genetic background of an individual affects susceptibility to disease and progression of disease. Also the response to drug treatment is determined by the individual's specific environmental and genetic settings. Different genes contributing to a specific phenotype may encode proteins involved in the same biological system or in its regulation. Therefore, causal genes in complex diseases

can be expected to affect the functionality of the same protein networks and pathways. If we can improve the prediction, identification and functional validation and characterization of networks involved in disease in carefully selected model systems and in humans, we will have a greatly increased likelihood of choosing the most reliable druggable targets for drug development. This will increase the chance of the drug to endure clinical development.

Current drugs to treat type 2 diabetes work by increasing β -cell insulin secretion, decreasing the amount of glucose released from the liver, increasing the sensitivity of cells to insulin, decreasing the absorption of carbohydrates from the intestine and slow emptying of the stomach to delay the presentation of carbohydrates for digestion and absorption in the small intestine. Drugs increasing insulin output by the β -cells have been widely used to treat type 2 diabetes and represent the existing group of diabetes drugs directly targeting the β -cells. These medications belong to a class of drugs called sulphonylureas, which increase insulin secretion by inhibiting ATP-regulated K^+ channels leading to plasma membrane depolarization and influx of Ca^{2+} that triggers insulin-containing vesicles to fuse with the plasma membrane and release insulin. Sulphonylureas are ineffective where there is absolute deficiency of insulin production as in type 1 diabetes. Development of novel drugs targeting the β -cell may represent new ways of increasing insulin secretion in type 2 diabetes and/or preserving β -cell mass and insulin secretory capacity in type 1 diabetes, see Box “The History of Insulin”.

How do we obtain a better knowledge of the pathological mechanisms, i.e. which protein networks and pathways that lie behind disease, and what kind of data can be exploited for this purpose? Much knowledge about disease mechanisms and pathologies is to a large extent based on data from animal models and cell systems. However, translation of results from animals and in vitro experiments to humans is often difficult due to the fact that the environmental and genetic settings of model systems are much too simple. Therefore, drug targets should preferentially be identified from a platform of human data. “Integrative genomics” is an emerging, promising field to tackle complex disease. It provides increased knowledge about functional mechanisms underlying disease and thereby an approach to increase our understanding of disease pathogenesis. Disease-associated networks today are, however, based on incomplete data; we have not yet characterized rare variation or copy number variation; we do not know enough about non-coding RNAs, alternative splicing, genetic isoforms, heterogeneity among populations, as well as dynamics in molecular systems. Most biological systems are characterized by considerable redundancy, and therefore the analysis of genes and proteins in the context of their networks will provide the most important functional and quantitative information. Networks should be seen as a framework of how to explore the context in which a given gene operates and to causally associate networks with physiological states associated with disease. This will lead to a more comprehensive understanding and view of disease as compared to examination of individual components of the network. Integrating data like DNA variations, gene expression data, DNA–protein binding and protein–protein interactions and molecular phenotypic data may construct more comprehensive networks and thereby improve understanding of the molecular processes underlying disease.

The History of Insulin

Insulin is the major therapeutic modality in type 1 diabetes, but today also a substantial part of type 2 diabetic patients are treated with insulin.

The Main Forerunners of Insulin

- 1848 Claude Bernard: Defined the normal values of blood glucose, the glycogenic function of the liver and the central regulation of blood glucose.
- 1869 Paul Langerhans: Publishes his description of the pancreatic islets, which later came to bear his name, but he was unable to explain their physiological role
- 1877 Étienne Lancereaux: Based on clinical and anatomopathological features he made the first correlation between the pancreas and diabetes (“the pancreatic diabetes”). He also made the distinction between “thin diabetes” (type 1) and “fat diabetes” (type 2)
- 1889 Joseph von Mering and Oskar Minkowski: Were the first to succeed in removing the pancreas and demonstrating that thereby permanent diabetes was produced. So, they confirmed experimentally what Lancereaux sustained based on clinical observations
- 1892 Edouard Hédon: Carried out stepwise ablation of the pancreas; he showed that “autograft” of a piece of pancreas prevents diabetes, and by cross-circulation he indirectly proved that the normal pancreas produces a hypoglycaemic substance
- 1893 Gustave-Édouard Laguesse: Drew proper attention to the almost forgotten observation by Langerhans, which suggests that the interacinar cells (which Laguesse designated “islets of Langerhans”) were a gland of internal secretion within the pancreas

The Discovery of Insulin

- 1919 Israel Kleiner: Documented the presence of a blood glucose lowering principle in pancreatic extract
- 1921 Nicolas Paulescu: Demonstrated that pancreatic extract caused lowering of blood glucose and glucosuria. Also described the physiological characteristics and the pharmacodynamic profile of this anti-diabetic hormone that he called “pancreine”
- 1922 James B Collip: Purification of the pancreatic extract used for the first successful clinical trial
- 1922 Frederik Banting: Experimental confirmation of the hypoglycaemic effect of the pancreatic extract in dogs. Awarded the Nobel Prize for the “Insulin Discovery” in 1923. Banting shares his award with Best
- 1922 Charles Best: Participation in many experimental and clinical applications of insulin
- 1922 John JR McLeod: He led the work in Toronto and took part in the clinical application of insulin. Named the hormone “insulin”, a name originally proposed by Sharpey-Schafer 1910 for chemical substance produced by the pancreas. Together with Banting awarded the Nobel Prize for the “Insulin Discovery” in 1923. McLeod shares his award with Collip
- 1955 Frederick Sanger: Description of the amino acid sequence of insulin; this is the first protein structure to be defined. This achievement earned him the Nobel Prize in 1958
- 1982 Using recombinant DNA technology, the first biosynthetic human insulin that is identical in chemical structure to human insulin was developed and can be mass produced

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Added by the editors

17.2.1 GWAS and Systems Biology

That diabetes has a strong genetic component is underlined by the fact that the concordance rate for both type 1 and type 2 diabetes is up to $\sim 70\%$ in monozygotic twins [2, 3]. Genetic variation may influence protein networks and thus cellular function at several different levels. Changes in amino acid sequence, alterations in protein expression or modification in enzymatic activity, etc. can be the result of genetic variation. Such changes to proteins can cause perturbations of the functionality of protein networks. Depending on the degree of disturbances of network function, this can lead to cellular malfunctioning, to changes in phenotype, and ultimately to disease. However, genetic variation may account for different levels of risk for disease in different individuals, suggesting that integrative methods for gene discovery are necessary. With the advent in recent years of huge amounts of data from genome-wide association studies (GWAS), transcriptomics and proteomics experiments, etc., now increasing focus is on interactions between DNA, RNA and proteins and whole-system physiology, as well as integration of large-scale, high-throughput molecular and physiological data with clinical data. Genome-wide association studies in complex diseases are producing an unprecedented amount of genetic data. However, identifying the individual genes can be difficult because each only contributes weakly to the pathology. Alternatively, identification of entire cellular systems involved in a particular disease could be attempted. Such a strategy should be feasible in many different complex diseases since most genes exert their function as members of molecular networks where groups of proteins contributing to disease may be expected to affect the same biological pathways. Experimental evidence for this is supported by the finding that the expression of genes which are all involved in oxidative phosphorylation is coordinately downregulated in human diabetic muscle [4]. Analysis of an entire disease-related biological system might provide insight into the molecular aetiology of the disease that would not emerge from isolated functional studies of single genes. It is clear that results of, e.g., GWAS do not themselves directly identify clinically useful drug targets, but by integrating GWAS data with other types of data and more refined phenotyping, this may well be possible.

Genetic disease loci for diabetes typically only confers modest disease risk, and only for very few are the causal genes known. Even replicated disease associations do not provide clues about the functional roles of a given candidate gene. A genetic

association is not enough for drug development strategies. There is no doubt that additional functional support is needed such as evaluating potential causal genes in the broader biological context in which they operate. The most likely causal candidate gene for an association may or may not be the gene in closest proximity of the associated single nucleotide polymorphism (SNP). However, a combination of such knowledge with an evaluation of the biological function of the genes, e.g. in expressional profiling studies under disease-relevant conditions and in functional studies, may provide insight into the mechanistic nature of complex traits beyond what human genetic association studies can do alone. Use of molecular traits can enhance the interpretation of GWAS results by putting them into a broader biological context and ultimately elucidate the networks defining disease-associated processes.

17.2.2 Moving from Genomes to Networks

If genetic data are integrated with networks of physically and functionally interacting proteins, this is likely to increase the probability of identifying positional candidate disease genes and proteins (Fig. 17.1).

Many disease-associated loci are known today; now the challenging task is identify the causal variants and to understand how they affect disease risk and

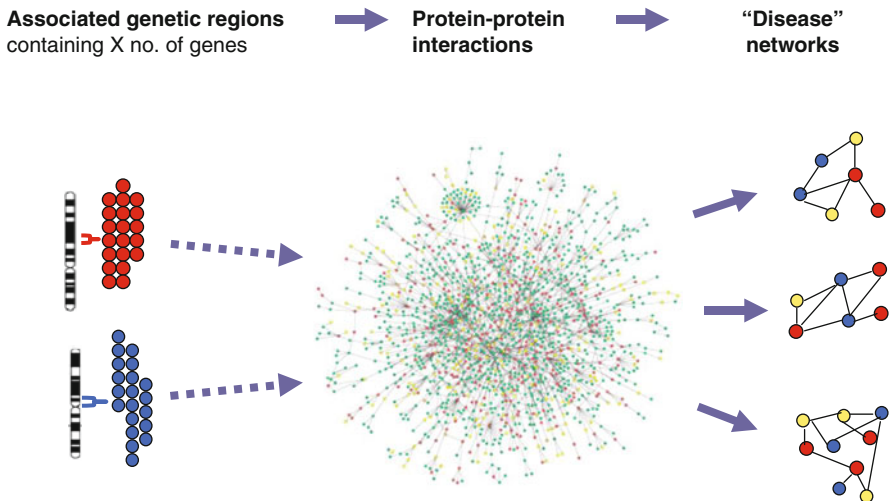


Fig. 17.1 Mapping of genetic loci onto a human interaction network. The creation of networks based on protein–protein interactions of proteins encoded by genes in genetic regions associated to disease allows identification of “disease” networks, i.e. networks that are enriched for proteins encoded by genes in these regions.

how to select key proteins for drug development. As mentioned, diabetes involves multiple interacting genetic determinants, representing functional relationships between genes, in which the phenotypic effect of one gene may be modified by another. However, new strategies for detecting sets of marker loci, which are linked to multiple interacting disease genes, are in demand. Data mining methods have been used to evaluate genetic interactions [5], and the importance of predicted genetic interactions was in this report supported by comprehensive, high-confidence protein–protein interaction networks of the corresponding regions. This allowed identification of candidate genes of likely functional significance in type 1 diabetes, representing a suggestion of genetic epistasis in a multi-factorial disease supported by protein network analysis with implications for functionality [5, 6]. Another approach for selecting candidate genes of functional importance is transcriptional profiling. Intermediate between DNA variations and variation in phenotype are variations in gene expression, protein expression, protein state and metabolite levels. Such intermediates are believed to respond to variations in DNA and then potentially lead to changes in phenotype and disease state.

Following identification of disease genes there is a huge demand for functional genomics. The number of identified susceptibility genes may continue to grow, and the elucidation of their function in the pathogenesis of diseases will be important for understanding their molecular pathogenesis. Approaches used will vary according to the function of the genes, but may include expression studies and generation of transgenic and knockout animal models. Whereas the genome is rather static, interaction networks are more dynamic and dependent on the biological context. They might be active only under certain conditions in certain cell types or stages of development. Ideally, all conditions and cell types should be tested to capture this presumed variability.

For prioritization of positional candidate genes in genetic association or linkage intervals the use of functional interaction networks (interactomes) may be a valuable method. If intervals obtained for a disease are queried for functional interactions with each other and related to phenotype information for the disease, this holds promise for selection of putative disease genes for further investigation [7, 8]. Such studies have the potential of identifying new, previously unrecognized components of disease mechanisms, as well as of pinpointing the most important protein complexes involved. Furthermore, many diseases have overlapping clinical manifestations/sub-phenotypes, and it could be speculated that this may be represented by genetic variation in the same functional pathways. The existence of the so-called disease sub-networks has been suggested. It was demonstrated that proteins encoded by genes mutated in one inherited genetic disorder were likely to interact with proteins known to cause similar disorders, presumably by sharing common underlying biochemical mechanisms [7]. The feasibility of constructing such functional human gene networks has been demonstrated and applied to positional candidate gene identification [9]. It was shown that obvious candidate genes

are not always involved and that taking an unbiased approach in finding candidate genes, e.g. by using functional networks, may result in new and better testable hypotheses [9].

17.2.3 Moving from Networks to Phenotypes

A systematic, large-scale analysis of human protein complexes comprising gene products implicated in many different categories of human diseases has been used to create a “phenome–interactome network” [8]. This was the first study to explain disease phenotypes by genome-wide mapping of genetic loci onto a human interaction network. This strategy was expanded to include epistasis and statistical methods for evaluating the significance of deduced networks [5]. Protein interaction networks were by this method used to examine whether gene products from interacting genetic regions could also be shown to interact in biological pathways. Support for physical interactions at the protein level for all the predicted genetic interactions was suggested [5], representing a novel exploration of integrative genomics. The resulting networks point directly to novel candidates visualized in the context of their interaction network, potentially providing even further biological insight. Another study evaluated changes at the proteome level after exposure of pancreatic insulin-producing cells to pro-inflammatory cytokines resembling the inflammatory milieu surrounding the islets in type 1 diabetes. That study demonstrated a large protein interaction network containing many of the differentially expressed proteins [10]. Despite the use of different species and model systems and unknown dynamic differences in the transcriptome and proteome, a significant overlap existed between genes pinpointed in this study [10] and in other studies [5, 6], providing evidence that common networks and pathways can be identified using different model systems and underlining the power of integrating protein–protein interaction data with genetic data and expression profiling.

Major histocompatibility complex (MHC) fine-mapping data have been analysed by the same approach to characterize the MHC susceptibility interactome [11]. This approach allowed identification of functionally important genes and gene–gene interactions independent of the genetic linkage disequilibrium that characterizes the MHC region, as protein–protein interactions are unlikely to depend on linkage disequilibrium between the genes encoding the proteins. Approaches like these may be valuable in prioritizing candidate genes in linkage regions or from disease-associated regions, in which the disease gene(s) are not known. Information on whether genes from the different loci observed do interact at a functional level is potentially interesting. Obviously, the input information is crucial for the success of such an approach. Studies will be biased by absence of complete functional information in databases of the majority of genes, and also interaction databases are far from complete. However, hypotheses generated with existing knowledge may be of value, and genes that would otherwise not have been predicted to be involved in the disease in question might be identified this way. Data amounts in databases are rapidly

increasing. This include increased knowledge regarding genes, proteins, interactions among them, methods integrating high-throughput genomic and proteomic approaches, as well as text mining methods extracting functional relationships from the literature.

Candidate genes involved in putative interaction networks should be further examined not only at the single-gene level but also in the context of the networks of which they form an integral part. mRNA expression levels for each gene can be evaluated, e.g. under different relevant conditions. Genes with differential regulation are believed to be most important. This approach has been used recently evaluating predicted interaction networks in type 1 diabetes [6]. Differential regulation of several genes was demonstrated, e.g. after cytokine exposure of human pancreatic islets, supporting the prediction of the interaction network as a whole as a risk factor. In addition, enrichment of type 1 diabetes-associated SNPs in the individual interaction networks was measured to evaluate evidence of significant association at network level. This method provided additional support, in an independent data set, that some of the interaction networks could be involved in type 1 diabetes [6].

17.2.4 Future Directions

Systems biology approaches complement more classical analyses of the genetics of complex diseases and may shed light on the underlying biological pathways and help us understand the complex interplay between multiple factors contributing to disease pathogenesis. Combining GWAS, protein networks, molecular biology studies and phenotype data in searching for functional candidates for observed genetic associations has been shown to be a feasible approach [5, 8]. Characterization of phenotypic effects of SNPs on gene expression or on protein function or interaction will provide a more efficient approach to the identification of risk variants and will provide insights into possible mechanisms whereby these variants modify disease risk. Focusing on interplay between many components in modules or systems may demonstrate how defects in such modules can lead to human disease. Such an understanding is likely to be helpful in defining new key targets for prediction, prevention and improved therapeutic responsiveness. Elucidation of networks and signalling pathways associated with disease and examination of the effects of combinations of experimental changes and variations are important in drug discovery and a prerequisite in translation of results into clinically useful predictors of disease and drug targets. Interaction networks can identify sub-networks corresponding to functional units in the biological system. Sub-networks associated with disease may link molecular biology to physiology and thereby to clinically relevant issues, and the aim is that predictive gene networks can lead directly to discovery of drug targets and biomarkers of disease.

For identifying drug targets it is necessary to understand how the causal genes function and act in their biological context. Identified genes from a GWAS may not be chemically suitable as drug targets. However, proteins in the same signalling pathway may constitute more rational and better drug targets. Disease-associated

genetic loci and intermediate molecular phenotypes that are connected with these loci and cause disease are obvious starting points to uncover the drivers of disease. It is important to evaluate perturbations of networks and pathways with the potential to thereby identify key steps or nodes that drive diseases, and which may act as targets for therapeutic intervention. To develop disease therapies by targeting a given gene it is necessary to know if activation, inhibition or partial activation leads to disease [12]. We can now begin to understand the context in which a gene operates and thereby suggest the best possible points of therapeutic intervention [12] (Fig. 17.2).

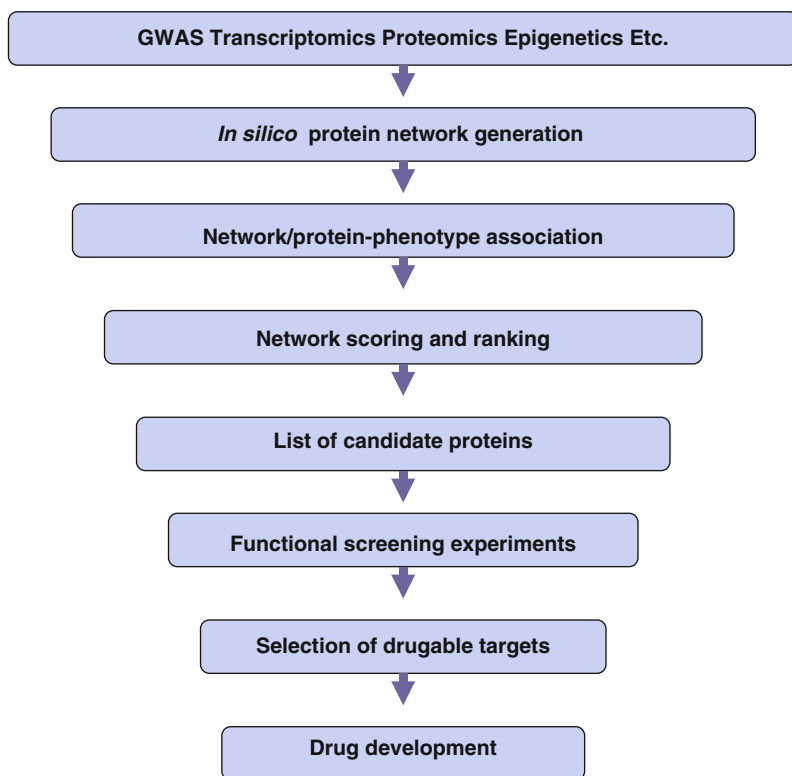


Fig. 17.2 Strategy for drug target identification. Genome-wide association scan data alone or integrated with transcriptomic, proteomic or epigenetic data, etc. are used as “input” data. Protein–protein interaction data and the application of bioinformatics and systems biology allow in silico generation of networks. Text mining analysis of these networks for enrichment of proteins with association to disease phenotype leads to a score and ranking of each network. This will end up in a list of potential candidate proteins whose functional relevance can be tested in model systems using, e.g., RNA interference. From the outcome of the functional studies, the most promising druggable targets are selected for drug development. Seen as a whole, this method will from a platform of thousands of data step by step narrow down the number of candidate proteins ultimately resulting in identification of a few numbers of plausible drug targets.

Clinical Trials

Clinical trials involving new drugs are commonly classified into four phases. Each phase of the drug approval process is treated as a separate clinical trial. The drug-development process will normally proceed through all four phases over many years. If the drug successfully passes through phases I, II and III, it will usually be approved by the national regulatory authority for use in the general population. Phase IV are “post-approval” studies.

Phase I trials are the first stage of testing in human subjects. Normally, a small (20–100) group of healthy volunteers will be selected. This phase includes trials designed to assess the safety, tolerability, pharmacokinetics and pharmacodynamics of a drug.

Phase II trials are performed on larger groups (20–300) and are designed to assess how well the drug works, as well as to continue phase I safety assessments in a larger group of volunteers and patients. When the development process for a new drug fails, this usually occurs during phase II trials when the drug is discovered not to work as planned, or to have toxic effects.

Phase III studies are randomized controlled multicentre trials on large patient groups (hundreds to thousands) and are aimed at being the definitive assessment of how effective the drug is, in comparison with current “gold standard” treatment.

Phase IV trials involve the safety surveillance and ongoing technical support of a drug after it receives permission to be sold. Harmful effects discovered by phase IV trials may result in a drug being no longer sold or restricted to certain uses: examples involve the anti-diabetic drugs phenformin and troglitazone.

Adapted from http://en.wikipedia.org/wiki/Clinical_trial#Phases.

Further Reading:

Chow S-C, Liu J-P (2004) Design and analysis of clinical trials: concepts and methodologies, 2nd edn. Wiley, New York, NY

Added by the editors

Systems biology approaches to develop drugs to treat human diseases is of high interest, and with the high cost of developing novel therapies, improved ways of selecting valid drug target candidates are extremely important. Novel and highly interdisciplinary systems biology approaches are likely to identify networks from which the most rational target can be selected. We are still far from a comprehensive understanding of the molecular pathogenesis of multi-factorial diseases. This makes it difficult to identify optimal strategies for intervention and treatment. The recent success of GWAS and the prospects for combining genetics with high-throughput genomics, as well as general advances in genome informatics, genotyping technology, statistical methodology and large clinical materials, are sources of optimism for the future.

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Chapter 18

Nanotoxicity

Gary R. Hutchison and Eva M. Malone

Abstract Nanotechnology, including the field of nanomedicine, promises to revolutionise/improve the way in which we live our lives. This atomic, molecular and macromolecular technology will introduce nanoparticles into our environment and our daily routine. In response to this exciting technology, toxicologists have responded with the development of a specialised subcategory of toxicology, nanotoxicology. This chapter will introduce nanotechnology and nanoparticles and examine the origins of nanotoxicology, drawing on epidemiology and respirable particle toxicology studies. These studies provide the foundation of what we today use to understand the toxicology of engineered nanoparticles. The focus of the chapter will be on human and mammalian nanotoxicology and will summarise the current understanding of the mechanisms of nanoparticle toxicity while describing the methodologies utilised to further knowledge in the area. In the context of the book, this chapter will briefly examine the impact of nanotechnology and the development of nanomedicines in relation to the pancreas. Nanomedicine relies heavily on nano-specific toxicological concepts and findings to provide safe medical applications. Success in this area requires a collaborative approach involving physicians, material scientists and toxicologists.

Keywords Exposure routes · Hazard assessment · Mechanistic nanotoxicology · Inflammation · Experimental models

18.1 Nanotoxicology and Nanoparticles

Nanotoxicology, as proposed by Donaldson et al. [21], is a subcategory of toxicology focused on the effects of materials in the nanoscale on living organisms and the potential, or likely, problems caused by these materials. This new area of toxicology

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has developed because substances ordinarily innocuous can have adverse effects in the nanoscale [39, 60]. The definitions for the terminology related to nanotoxicology have evolved over time, and there is some conflict in the literature, so, at this stage, it is important, for clarity, to discuss some of the current terminology related to nanotoxicology. The British Standards Institution (BSI) has defined nanoscale as a “size range of approximately 1–100 nm” (PAS 136 [4]). The National Nanotechnology Initiative (NNI) also uses the term nanoscale to describe dimensions between approximately 1 and 100 nm (<http://www.nano.gov/html/facts/WhatIsNano.html> accessed on the 8th December 2009). Nanoparticles are defined by the BSI as discrete pieces of material with all three external dimensions in the nanoscale (PAS 136 [4]). This definition is generally accepted and will be the definition followed in this chapter. However, the BSI had previously defined nanoparticles as “discrete pieces of material with one or more external dimensions in the nanoscale” (PAS 71 [3]). The term now being used to describe this material is nano-object (PAS 136 [4]), for example nanotubes. In addition, the term ultrafine particle has been in use for some time to denote nanoparticles. The term nanoparticle is a relatively new term. It is also important to be aware that nanomedicine can relate to nanometre size complexes from one to hundreds of nanometres in size [24]. Nanoparticles do not generally exist as discrete uniform entities; they can form agglomerates and aggregates through chemical and physical interactions. Furthermore, they come in a variety of different shapes and sizes. For example, high aspect ratio nanomaterials (HARN), which have a diameter in the nanometre range, can range in length from nanometre to hundreds of microns. Thus the material exists as long, thin particles; however, not all high aspect ratio nanomaterials are nanoparticles. Compositions can also vary, from individual elements, for example nano gold, to complex engineered structures, such as quantum dots which can have core made from one element and outer shells made of other elements. Nanoparticles can also be engineered to be functionalised for a desired purpose. Therefore it is naive to assume that all nanoparticles will be similar and behave in a generic manner.

Throughout evolution humans have been exposed to naturally occurring nanoparticles, for example, viruses and nanoparticles generated by active volcanoes. In addition to naturally occurring nanoparticles, there are anthropogenic nanoparticles, those generated unintentionally, for example, by internal combustion engines, power plants, incinerators, automobile and jet engines, and those generated intentionally including metals, metal oxides and carbon [47]. Historically, particle toxicology has been connected to industrial materials, for example coal and asbestos [15], and research has focused on anthropogenic nanoparticles generated unintentionally, for example, the ultrafine (diameter less than 100 nm) respirable air pollution particles of PM₁₀ and particulate air pollution with aerodynamic diameter of less than 10 μ m (reviewed by Stone et al. [63]).

The number of nanoparticles generated intentionally, or engineered, has increased as a result of the rapidly developing field of nanotechnology, an important research endeavour of the twenty-first century, which has been described as molecular manufacturing, one atom or molecule at a time, and includes research and development technology of nanoparticles [1, 43]. The Project on Emerging

Nanotechnologies (PEN) provides an extensive and diverse list of nanotechnology-based products currently available to consumers. Those on the list include cosmetics, sunscreens, sports clothing, electrical appliances and food packaging products (<http://www.nanotechproject.org/inventories/consumer> accessed on the 8th December 2009). Nanoparticles are also being used for drug delivery, in vivo imaging and in vitro diagnostics as nanoparticles operating at the biomolecular scale; human cells are 10,000–20,000 nm in diameter, a 100–200 times the size of nanoparticles. Their size, in combination with their surface tailorability, solubility and multifunctionality, offers biologists the opportunity to target, diagnose and treat diseases. However, the benefit of nanoparticles must be balanced with the potential health risks associated with the manufacture, distribution and use of nanoparticles [43]. The focus of the chapter will be on human and mammalian nanotoxicology.

The first rule of toxicology is that all substances produce an effect, but it is the dose that decides whether the effects are adverse or beneficial (Paracelsus). Before exposure routes and toxicity can be discussed it is important to understand the relationship between hazard and risk. There are three factors that require consideration when making hazard/risk assessment: hazard, risk and exposure. A hazard exists when a substance, object or situation has an intrinsic ability to cause an adverse effect. Risk, on the other hand, is the chance that such effects will occur: the risk can be high or negligible. These alone do not allow a complete assessment. For harm to occur in practice, in other words, for there to be a risk – there must be both the hazard and the exposure to that hazard. Without hazard and exposure there is no risk. Regulatory, public health organisations and industries all recognise the value of the toxicological triage process that accompanies hazard classification and risk assessment. In the field of nanotechnology, nanotoxicologists are tasked with collating the necessary hazard information for the new nanoparticles that are currently being produced by the nanotechnology industry. In order to advance our understanding of acute and chronic nanoparticle toxicity and nanoparticle translocation, biodegradation and elimination from the body, we must first balance and prioritise efforts to understand how likely nanoparticles are to gain access to the human body (Donaldson 2006).

18.2 Potential Routes of Exposure

Human exposure to nanoparticles can be described as incidental, accidental or deliberate. For example, human exposure to engineered nanoparticles, manufactured for a specific purpose, for example nanomedicines, can be intentional or deliberate. Exposure to engineered nanoparticles can also be incidental or accidental, for example nanoparticle release during nanoparticle manufacture, or use, can result in occupational, consumer and/or environmental exposure. Exposure can result from natural sources, for example nanoparticle release from forest fires or anthropogenic nanoparticles generated unintentionally [2]. Figure 18.1 provides a summary of the possible routes of human exposure to nanoparticles, potential interactions and the

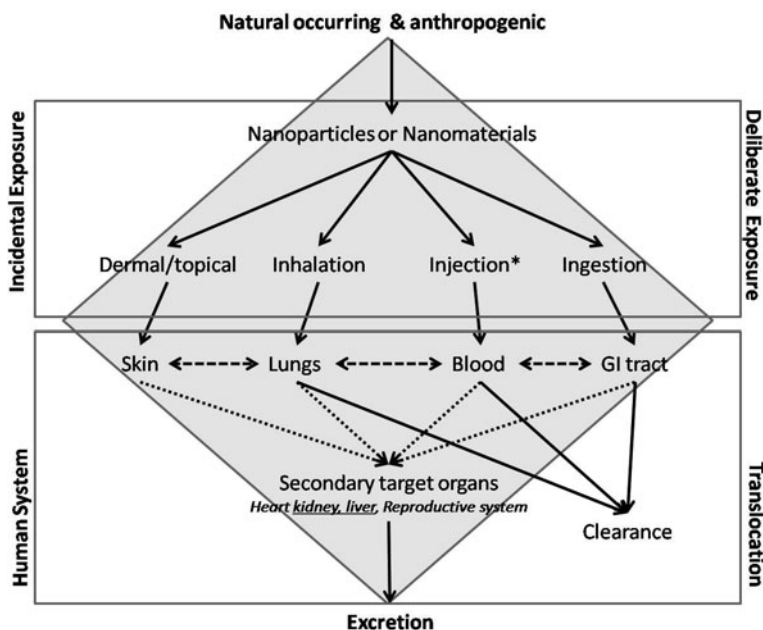


Fig. 18.1 Potential routes of human exposure to nanoparticles either via deliberate, incidental or accidental exposure. *Arrows* depict potential routes of travel through the body with nanoparticle clearance via the lung, mucociliary escalator, gastrointestinal (GI) tract and lymph. As *underlined*, excretion can occur by route of the kidney or liver. *Solid arrows* depict known routes of exposure. *Dashed lines* between target organs indicate that there is potential for translocation between primary target organs. *Dotted lines* depict the possible secondary target organs that could be exposed to nanoparticles if translocation occurs. * denotes that deliberate injection can be subcutaneous, intramuscular and not just intravenous.

possible clearance routes of nanoparticles from the body. For example, it is well documented that inhaled nanoparticles deposit within the alveoli [26, 41] and can subsequently be cleared by alveolar macrophages via the mucociliary escalator and expelled by the nose or swallowed. Alveolar macrophage transport of nanoparticle from the lung could potentially lead to translocation of nanoparticle to other organs within the body such as the gastrointestinal (GI) tract and or lymphatic system via macrophage migration to the local draining lymph nodes.

Nanoparticle exposure is not only a human health concern as they equally effect the environment [27]. Nanoparticles have also been utilised in environmental remediation/waste treatment; free nanoparticles are added to contaminated environments in an attempt to clean up soils and/or groundwaters from organic and inorganic pollutants.

Nanoparticles have many potential diverse applications, and therefore there are a number of expected exposure routes associated with nanoparticle utilisation and production: specifically inhalation, intravenous injection, ingestion and dermal application (Oberdorster, et al. 2008).

Time is also an important parameter when considering exposure in a relevant population. It is important to understand whether a population is exposed over a long period of time, i.e. chronic exposure, or whether a population comes into contact with the material for short periods of time, i.e. acute exposure. It is expected that workers in the nanotechnology industry, generating nanoparticles or using nanoparticles, will be exposed to higher concentrations than consumers of, or recipients of, nanotechnology-based products. Inhalation and dermal routes of exposure are potentially greater for workers in the nanotechnology industry. Consideration of the dose, route of exposure and exposure period has a significant bearing on the hazard/risk assessment of material in the nanoscale. For example, an employee in a factory may be exposed to high levels of a relatively inert nanoparticle, via inhalation and dermal routes of exposure, over a prolonged period of time. Therefore, the concentration of nanoparticles entering the body could increase over time and persist in the body resulting in a pathogenic effect. This pathogenic effect may not materialise in a consumer population if consumers are exposed, by dermal routes only, to low levels of the relatively inert nanoparticle over a short period of time.

18.2.1 Portals of Entry

Potential routes of nanoparticle exposure in the human population include the respiratory tract, skin, GI tract and in the case of biomedical applications possibly injection into the body, for example, intravenous, subcutaneous or intramuscular injections. Any potential adverse effects resulting from nanoparticle exposure may occur at the various portals of entry, the primary target sites or organs, such as the lungs and skin; however, it is possible that the adverse effects may occur at distant sites, or secondary target organs such as the kidney or liver (after nanoparticle translocation from the primary organ). For prediction of systemic toxicity, following nanoparticle exposure, systemic dose is another important parameter to consider. The systemic dose is dependent on both the barrier function and the clearance mechanisms at the portals of entry. It is postulated that respirable particulate air pollution or induced mediators from the lung are able to cross into the circulation, via the alveolar epithelium, and induce fibrogenic plaques in the cardiovascular system. Epidemiological studies support these findings as hospital admissions increase during episodes of high air pollution. Predominantly these adverse health effects are manifested in susceptible individuals who had pre-existing pulmonary or cardiovascular disease [14, 52, 58]. Studies addressing the systemic translocation of nanoparticles from primary sites of deposition are beginning to unravel the dynamics of nanoparticle–organism interaction and provide the means to relate exposure to hazard data. Nanoparticle translocation is a challenge for nanotoxicologists as current techniques are not sensitive enough to track all nanoparticles *in vivo* and to detect and measure nanoparticle concentrations *ex vivo*. Current techniques need to be adapted, or new techniques developed, to allow nanoparticle translocation to be assessed for all nanoparticles.

It is also important to mention that not all nanoparticles that enter the body will deposit and accumulate within primary and secondary organs. Some nanoparticles can be degraded by the body's defence mechanisms, e.g. alveolar macrophages can readily clear inhaled particulate from the lung which is subsequently cleared via the mucociliary escalator. Additionally, a number of studies have shown that some nanomaterials are readily excreted via the kidneys or GI tract. From a nanotoxicologist's perspective, removal of nanoparticles from the body, by the body's defence mechanisms, may be an ideal outcome; however, a nano-engineer, producing a nanomedicine designed to biopersist, may consider this less than ideal.

18.3 Historical Perspective

Historically, as mentioned previously, nanoparticle research has focused on the ultrafine component of PM_{10} . PM_{10} is the commonly applied international standard given to environmental particulate air pollution that measures the mass of particles collected, with a 50% efficiency for particles with an aerodynamic diameter of $10\ \mu m$ [42]. PM_{10} can be divided into three categories based on size: coarse (PM_{10} – $PM_{2.5}$), fine ($PM_{2.5}$ – $PM_{0.1}$) and ultrafine (nano) ($<PM_{0.1}$) [20]. PM_{10} is made up of a number of different components including fine and nanoparticles from combustion sources, transition metals, sulphates and nitrates, as well as biological components, for instance, pollen, endotoxin and wind-blown dusts such as clays [61], and these components can vary between different PM_{10} samples. The association between adverse health effects and increased levels of particulate air pollution has been well documented, with more than 100 epidemiological studies published since 1992 [23, 28] (Pope and Dockery 1999). Although air pollution levels have gradually decreased since the late 1950s due to the introduction of “Clean Air Acts”, the international community has moved away from solid fuels, which release high levels of sulphur dioxide to oil and petroleum resulting in a progressive increase in levels of ozone, hydrocarbons, oxides of nitrogen and PM_{10} [13]. Since the publication of the WHO Air Quality Guideline for Europe in 1987 [10] and subsequent updates [11] many epidemiology studies have shown that PM_{10} levels above the set guidelines have caused adverse health effects [8]. These effects are reviewed in Pope et al. (1995) and demonstrate a clear relationship between increased levels of PM_{10} and exacerbations of asthma and chronic pulmonary disease (COPD). Deaths, not only from respiratory causes but also from vascular causes, i.e. myocardial infarction and cerebrovascular accidents, are also related to levels of PM_{10} [68]. Air pollution is increasingly recognised as an important and modifiable determinant of cardiovascular disease in urban communities. Acute exposure has been linked to a range of adverse cardiovascular events including hospital admissions with angina, myocardial infarction, and heart failure. Long-term exposure increases an individual's lifetime risk of death from coronary heart disease. The main arbiter of these adverse health effects seems to be combustion-derived nanoparticles that incorporate reactive organic and transition metal components. Inhalation of this particulate matter leads to pulmonary inflammation with secondary systemic effects or, after

possible translocation from the lung into the circulation, to direct toxic cardiovascular effects. The Utah study by Pope and colleagues [53] was ground breaking in linking air pollution, particularly PM₁₀, with adverse health effects. The Utah scenario was a landmark study as it is unusual for an environmental intervention study to take place where the source of the pollution is switched off and switched on again, allowing researchers to examine clearly the effects of air pollution, providing a unique opportunity to examine the health effects of PM₁₀ in humans. The temporary closing of the steel mill provided researchers with the unique opportunity to demonstrate a correlation between exposure to PM₁₀ and observed health outcomes. Schwartz [59] posed the question whether studies associating air pollution with increased hospitalisation and mortality rates could in fact be confounded by influenza or other illness and thus suggested that there may be a “harvesting effect” when it came to data collection. Schwartz’s study concluded that deaths increased only for patients outside the hospital who were exposed to increase levels of air pollution, while death rates for patients not exposed to the outside air remained consistent. Toxicity studies have provided evidence of the toxicity of many nanoparticles, but epidemiological proof for health effects of nanoparticles is limited. As such there is currently no data available to make concentration–response functions for nanoparticles that can be used in health impact assessment [31].

18.4 Nanoparticle Toxicity

The assessment of nanoparticle toxicity can include *in vivo* studies or *in vitro* testing with nanoparticle dose being an important consideration. Artificially high doses are often used in initial, proof-of-principle, *in vivo* studies to induce a toxic effect, so that potential toxicity can be ascertained, while limiting the number of animals used. High doses can also be used in *in vitro* studies to induce a toxic effect, for example, 100 µg nanoparticles/ml of cell culture supernatant. These studies should be followed up with realistic *in vivo* doses [47]. However, not all nanoparticles are toxic, and some are used successfully as drug delivery vehicles [43], but toxicity testing is still required for the majority of nanoparticles.

The delivery of nanoparticles to animals in *in vivo* studies can involve a variety of methods. The method of exposure is usually selected based on the proposed or suspected route of entry, albeit incidental, accidental or deliberate, into the body. Traditionally particle studies involving animals have focused on the lung as the primary exposure site and can involve inhalation studies and/or instillation studies. Inhalation studies involve inhalation of small doses, delivered daily, over various timescales (ranging from days to years) while instillation studies involve the instant exposure of the animal to a relatively high dose of the particle instilled into the lung [18]. However, due to the possibility of nanoparticle accumulation in the food chain and/or possible nanoparticle ingestion, nanoparticle toxicity testing can include intragastric dosing where particles are introduced into the stomach [29]. Nanoparticles are also being used in formulations for topical use, and therefore dermal toxicity studies are being utilised to assess their safety [34]. Additionally,

particles utilised in nanomedicine may be deliberately injected into the bloodstream, and therefore this route of entry should be considered in nanoparticle toxicity testing, when appropriate. Ultimately, the *in vivo* studies are carried out to ascertain the adverse effects of nanoparticle exposure on, not collectively or exclusively, the immune system, cardiovascular system, nervous system and digestive system.

In general, nanoparticle toxicity testing uses *in vitro* methods. There are several advantages for *in vitro* toxicity testing, and they include their relative low cost compared to *in vivo* studies and the speed at which the testing can be completed and from an ethical perspective, the reduction of animal testing. It is important to state that there are limitations and challenges with using *in vitro* assays to perform nanoparticle risk assessments as it has become apparent that nanoparticles can interfere with the current *in vitro* testing methods (reviewed by Kroll et al. 2009). Additionally, *in vitro* systems cannot fully replace the complex interactions that occur *in vivo* within and between organs and cannot be used for true pharmacokinetic or toxicokinetic studies. However, it has been estimated that hundreds or thousands of animals would be required to ascertain the potential hazard of each nanoparticle under development or production; therefore, there is a need for toxicologists to develop effective *in vitro* assays for the assessment of nanoparticle toxicity [64].

Primary cells isolated from animals or humans can be used for *in vitro* tests. They correspond closely to the cells *in vivo*, but they have a limited lifespan *in vitro*. Rodent cells are used in most instances as human cells can be difficult to obtain. Cell lines are more commonly used because they have an unlimited lifespan *in vitro* under the appropriate conditions. However, they are transformed cells and therefore do not correspond as closely to the cells found *in vivo* as primary cells.

The first step of an *in vitro* study should be to determine the cytotoxic potential and LC₅₀ (lethal concentration, 50%) of the nanoparticle of interest using toxicity or viability assays, of which there are a wide variety [64]. When sublethal concentrations have been determined these can be used in subsequent tests analysing cell function, for example. *In vitro* toxicity testing can also involve the assessment of reactive oxygen species (ROS) production by nanoparticles or by cells exposed to nanoparticles. ROS are oxygen-containing molecules that have unpaired electrons and therefore are highly reactive. They can damage DNA, proteins and lipids, and production by cells can be indicative of a stress response (see Section 18.4.1.1). Additionally, as inflammation is central to any potential adverse particle effects *in vivo*, many steps in the process of inflammation can be analysed in response to nanoparticles *in vitro*, for example, calcium flux, non-specific stimulation of receptors and pro-inflammatory gene expression. Furthermore, cell death and oxidative stress are both stimulators of inflammation [18].

Currently, to effectively ascertain the potential hazards of nanoparticles, it is necessary to complete a combination of *in vitro* and *in vivo* studies [17, 47]. However, it is envisaged that scientists will develop a battery of *in vitro* tests to be used as alternatives to animal testing [64].

18.4.1 Mechanisms of Nanoparticle Toxicity

Much of the research into the mechanism of nanotoxicity has been conducted in the rodent lung. The small physical size of a nanoparticle means that there are an extremely large number of particles per mass unit. The inhalation of such small particles can result in large numbers being deposited in the lower alveoli. As a result, efficient phagocytosis and clearance via the macrophage system can be prevented due to high burden (rodent phenomenon) [46, 57]. Once in the lungs, nanoparticles can interact with epithelial cells, macrophages and neutrophils, whose activation results in the initiation, progression or prolongation of intracellular and extracellular signalling mechanisms that control inflammation. In the extracellular environment, inflammation is activated and controlled by a wide range of molecules such as cytokines [35], prostaglandins [49] and leukotrienes [30]. Pro-inflammatory cytokine expression has been a major focus when examining the impact and effects of pathogenic particles [5]. Ongoing research strives to ascertain whether any of the additional extracellular signalling pathways may play a role in the potential toxic effects of nanoparticles. The role of the intracellular signalling pathways, in response to nanoparticle exposure, is generally to control the production of the extracellular pro-inflammatory mediators described above. At this point it should be noted that nanoparticles, when in contact with biological systems, can be altered by adsorption of protein and other biological materials onto the surface of the particle, a subject covered in greater detail in [Chapter 9](#), [12]. This point is important for nanotoxicologists as it means that nanoparticles can change upon introduction into biological conditions. Therefore there is the need for full characterisation of nanoparticles and to understand that these characteristics may change, and in doing so may alter the behaviour of the raw material. A brief summary follows describing the current mechanistic knowledge of how nanoparticles can elicit a toxic response.

18.4.1.1 Oxidative Stress

Oxidative stress occurs when there is an imbalance between oxidants and antioxidants that favours oxidant presence, due to the excessive production of oxidants or depletion of antioxidants. Although nanoparticles are small in size they present a large surface area for the generation of free radicals as a result of redox cycling at the particle surface. [7] confirmed that particles of different composition, including carbon and polystyrene, generate ROS and were more inflammogenic than larger particles of the same chemical composition when added to lung cells. ROS are transferred to the interior of the cell where they can result in oxidative stress, activating transcription factors for pro-inflammatory mediators [7, 19, 22, 65]. Additionally, ROS can react with macromolecules such as lipids, proteins and DNA to compromise normal cell function, which can occur to such an extent that cell death results [40].

18.4.1.2 Calcium Flux

Nanoparticle carbon black, but not larger carbon black particles, has been shown to induce calcium influx in a monocytic cell line [69] and in primary macrophages [9]. Furthermore, polystyrene nanoparticles have the ability to induce calcium signalling in macrophages [56]. Calcium signalling induced by carbon black nanoparticle was shown to be important in activating the transcription factor NF κ B and production of the pro-inflammatory cytokine TNF α [6]. There is substantial evidence that ROS and oxidative stress can alter calcium signalling in cells. Cytosolic calcium is a key intracellular signalling molecule that controls a variety of cellular processes [33]. There is also substantial evidence from the literature that calcium signalling can control inflammation [26]. Taken together this evidence would suggest that ROS can modulate calcium signalling leading to the activation of an intracellular signalling cascade that results in transcription factor activation and the production of pro-inflammatory cytokines.

18.4.1.3 Inflammatory Response

The mechanism by which particles induce a pro-inflammatory response within the lungs is thought to be a consequence of the increased transcription of pro-inflammatory mediators regulated by increases in intracellular calcium and oxidative stress [62, 66]. In the lung, epithelial cells play an important role in the defence mechanisms of the body against foreign particles. They can generate chemotactic factors that recruit phagocytic and inflammatory cells, such as macrophages, when exposed to damaging or infective agents. Attention has focused on the pro-inflammatory effects induced by nanoparticles, due to the characteristic neutrophil-driven inflammatory response observed within the lungs of treated rats [41] and the associated increase in pro-inflammatory cytokine production, such as TNF alpha (TNF α), interleukin (IL)-8 and IL-1. Some nanoparticles have been shown to stimulate epithelial cells *in vitro* to produce the chemoattractant IL-8 [44] and nanoparticle carbon black induces glutathione depletion, in a human lung epithelial cell line, which is indicative of oxidative stress [65].

It is important to note that a majority of the *in vivo* and *in vitro* nanoparticle studies published over the last decade have used particles that were aggregated and/or agglomerated. In many of these studies, despite a reduction in the exposed particle surface area, there remains a clear relationship between particle surface area, inflammation and biological responses.

18.5 Nanomedicines for Pancreatic Disease

Nanotoxicology offers the potential to allow the safe development of new nanotechnologies and applications including those related to nanomedicine. Nanomedicine is still in its infancy; however, it has major potential applications in a range of

diseases and target organs including diabetes and the pancreas: for example, glucose monitoring, insulin delivery and diagnostic and imaging applications. New nano-encapsulation technologies, such as layer-by-layer biocompatible nanofilms, involving the implantation of stable electrodes offer the possibility of continuous glucose monitoring. Nanomedicines are also being developed to solve current therapeutic problems such as enhanced insulin delivery in diabetes by both improved islet encapsulation and oral insulin formulations. An “artificial nanopancreas” could be an alternative closed-loop insulin delivery system whereby animal islets or insulin-producing cell lines are in a permanent selective membrane that facilitates glucose-dependent insulin release and nutrient access across the membrane, while excluding the large proteins and cells of the immune system. As discussed in [Chapter 9](#), nanoparticles in the form of quantum dots or gold can be used in vivo to enhance targeted imaging. This technology could be used to monitor tissue complications, and single-molecule detection systems could be employed to the study of molecular diversity in diabetes pathology [50]. The technology is being used to aid visualisation of transplanted pancreatic islets, in doing so, advancing the use of islet transplantation for treatment of type 1 diabetes by helping to confirm technical success and to diagnose rejection. The literature highlights a crucial need for non-invasive assessment tools to monitor the success of cell transplantation. As discussed in [Chapter 9](#), several studies have investigated whether an improved magnetic resonance imaging (MRI) strategy, currently a non-invasive experimental cell-based monitor of therapy, could be clinically applied to islet transplantation. The current strategies available use positron emission tomography and MRI to image islets. Advancements to improve these strategies have involved the use of superparamagnetic iron oxide nanoparticles, as discussed in greater detail in [Chapter 7](#), to enhance labelling of cells and to visualise via MRI. Several studies have demonstrated that islet visualisation using superparamagnetic iron oxide (SPIO) is eminently applicable to islet transplantation even in humans [32, 67]. Feridex, a representative dextran-coated SPIO nanoparticle with a diameter of 70–140 nm, was extensively used for this purpose [25]. A new SPIO agent, Resovist, currently approved for clinical use as a liver imaging agent may also act as a labelling agent for islet transplantation. Resovist is composed of carboxydextran-coated iron core nanoparticles comprising multiple crystals, with an overall hydrodynamic diameter of 62 nm. It was tested in vitro and shown to enhance MRI imaging on islet viability, insulin secretion and gene expression. This suggests that the technology can image islets with no deleterious effect on islet function or gene expression [37]. Nanomedicines are also being developed to improve the diagnosis and treatment of pancreatic cancer. Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States [36]. The average survival of 6 months is due, in part, to limitations in the diagnostic procedures; therefore the disease can elude detection during its formative stages. Nanoparticle contrast agents have been developed to enhance the visualisation of very small pancreatic tumours. Although many good nanoparticle-based contrast agents have been developed, most of them work by targeting molecules expressed on the surface of tumour cells. Unfortunately, normal (noncancerous) pancreas tissue expresses many of these same molecules, making it

Classical Toxicity Studies

Dealing with poisons, their effects and methods of treating and preventing poisoning, counts among the oldest and most developed branches of human arts. Ancient Greek physicians knew of poisons and poisonings, as seen in the works of Dioscorides (first century of CE) and Galen. Applied toxicology was furthered by the works of Paracelsus and the emergence of iatrochemistry. As an academic discipline, however, it is rather young, established by the French physician M.-J.-B. Orfila, who in 1818 published a manual on *forensic toxicology*.

Last century witnessed the synthesising of ever more chemical compounds in ever greater amounts and/or ever wider spread. As a consequence, the focus of toxicology has moved away from investigating murder cases to general studies of the *toxicity of chemical compounds*: the dissemination in the environment and in living organisms, their accumulation in organs and tissues, their biological transformation, their elimination from the organisms and the nature of their adverse effects. Jointly with lawmakers, toxicologists are developing criteria for the biological effects of chemical pollution, systems of maximum permissible concentrations, methods for the toxicological standardisation of raw materials and food products and standards for clinical trials of new drugs (see Box “Clinical Trials” in [Chapter 17](#)).

To an outsider, classical toxicology can constitute an overwhelming impression due to its particular combination of *elaborated strict systematic* and *almost chaotic abundance of approaches and results*. In that, classical toxicology resembles very well the study of mathematical equations: they are so well structured, and sometimes, nevertheless, the most easily solvable equation is very close to intractable equations or equations which require radically different approaches. While many procedures in experimental sciences depend on the tacit assumption of *structural stability*, i.e. assuming that small deviations will seldom make big differences, it seems that the play of classical toxicology is different: roughly speaking, there is no scientific basis how to infer from chemical similarity to toxic similarity, from large dose to small dose, from in vitro tests to animal trials and further on to clinical trials, from acute to long-term tests, from macroscopic aggregates to nanoscale powders of the same substance. Some laymen are mystified and may ask why it took 80 years to discover the blood thinning property of aspirin – acetylsalicylic acid (as anti-clotting mostly benign for elderly people); why pigs can be nourished with white amanita, while a teaspoon of this mushroom kills a human; why cows are killed by grams and milligrams of dioxin, but, according to some reports, a human could eat a spoon full of it in self-experimentation (though with high cancer risk)?

The emerging wide field of *toxicogenetics* and the ongoing development of *nanotoxicity testing protocols* have their own agenda. One may expect that they will shed some light also on the preceding old, but common questions.

Further Reading:

Greim H, Snyder R (eds) (2008) *Toxicology and risk assessment – a comprehensive introduction*, Wiley, Chichester

Added by the editors

difficult to distinguish tumours from healthy tissue [36]. Montet et al. [45] devised an inverse targeting approach for imaging pancreatic cancer by designing nanoparticles which bind to the peptide bombesin, a ligand that will bind to receptor proteins only found on normal, as opposed to cancerous, pancreatic cells. Therefore the target, on the surface of normal pancreas cells, will be absent on PDAC [45]. Montet's studies in mice, injected with the nanoparticles conjugated to bombesin peptide, revealed that magnetic resonance images displayed tumours because they did not bind to the nanoparticles.

Effective drug delivery in pancreatic cancer therapy remains a major challenge. High resistance of tumours to chemo- and radiation therapy results in extremely low survival rates for pancreatic cancer. Recent advances using nanoparticles to deliver cancer drugs and other therapeutic agents, such as siRNA, suicide gene, oncolytic virus, small molecule inhibitor, have been a success in recent preclinical trials [70].

18.6 Summary

This chapter provides a summary of both historical and “state of the art” regarding studies of human and mammalian nanotoxicology. As yet there is no reason to think that all nanomaterials will induce toxic or harmful effects. Previous experiences with particle air pollution have provided a foundation for nanotoxicologists; however, it is now evident that applying standard toxicological tests can only provide a limited assessment of the potential hazards these new materials may illicit. The literature available describes the current methodologies and understanding of nanoparticle toxicity mechanisms. To advance the science, new or improved methodologies must be developed to allow a better understanding of how nanoparticles behave in situ.

The lack of information and research on nanomaterial toxicity is important to consider when assessing the potential health and safety issues. A hazard/risk assessment will have to be completed for every new nanoparticle, as is done for any new pharmaceuticals, diagnostics or medical materials. If done correctly it should allow a balanced approach to nanomaterial/medicine design and implementation.

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Part V
Mathematical Modelling and Numerical
Simulation

Chapter 19

From Silicon Cell to Silicon Human

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Abstract This chapter discusses the silicon cell paradigm, i.e. the existing systems biology activity of making experiment-based computer replica of parts of biological systems. Now that such mathematical models are accessible to *in silico* experimentation through the World-Wide Web, a new future has come to biology. Some experimentation can now be done *in silico*, leading to significant discoveries of new mechanisms of robustness, of new drug targets, as well as to harder validations or falsifications of biological hypotheses. One aspect of this future is the association of such live models into models that simulate larger parts of the human body, up to organs and the whole individual. Reasons to embark on this type of systems biology, as well as some of the challenges that lie ahead, are discussed. It is shown that true silicon cell models are hard to obtain. Shortcut solutions are indicated. One of the major attempts at silicon cell systems biology, in the Manchester Centre for Integrative Systems Biology, is discussed in some detail. Early attempts at higher order, human, silicon cell models are described briefly, one addressing interactions between intracellular compartments and a second trying to deal with interactions between organs.

Keywords Bottom-up systems biology · Computational · Networks · Modelling · In silico experimentation · Metabolic control · Pharmacokinetics and systems biology · Regulation

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19.1 Introduction

This chapter addresses how the molecular biology of cell types may be related to their cell biology and how both of these may be related to the functioning of a multi-cellular organism. It focuses on methodologies that make realistic models. These methodologies enable the understanding of mechanism and control of function. This analysis package is comprehensive, because the authors of this chapter have invested considerable effort to make it such. Although the endocrine role of insulin production by β -cells is what the authors have in mind, this application is not made explicit, in part because too little has been done and in part because this chapter wishes to inspire experts to have a fresh go at this.

We shall first address the differences that systems biology may make. Subsequently we shall describe multiple aspects of our silicon cell mode of systems biology. We end by speculating how the approach may lead to a true-to-life model of how the human being functions through its interacting molecules.

19.1.1 Where Systems Biology Is Different

Genomics and molecular biology have focused on the identification of all the individual macromolecules, their inherent activities, and sometimes their interactions with immediate partners. Molecular cell biology has drawn schemes that indicate which macromolecules interact with which other macromolecules, either directly or indirectly. Some of these schemes distinguish between stimulatory and inhibitory interactions. Few of them indicate the strengths of the interactions, and none of them indicate how the strengths of the interactions may depend on other factors, such as concentrations of other molecules in the network or the concentrations of the interactors. Probably in view of the robustness and adaptability of biological functions, the latter tend to be regulated through both positive and negative interactions. As a consequence one cannot come to understanding and predictions without assessing the strength of the interactions quantitatively. Because insufficient attention has been paid to collecting the intermolecular interactions data quantitatively and because all data relevant to a certain function have rarely been integrated into a single frame of reference, network analyses have remained qualitative and thereby speculative.

On the other hand, mathematical biology has had the tendency to abstract away from the detail and the actual, because it aimed for generic principles. Of the principles that were found, such as gradient-driven self-organization as possible mechanism for developmental biology, specific predictions could be falsified. This made self-organization theories irrelevant in the eyes of experimental developmental biologists [10, 22, 26]. As an alternative paradigm for developmental biology, the concept of the genetic program became popular, in which the expression of one gene would lead to a protein activating the expression of the genes of the subsequent phase. Although feedback and feedforward loops are

recognizable in the corresponding networks, it is not clear whether self-organization plays a role [26].

To understand living organisms we need to appreciate with sufficient precision how their components interact. We need to reckon with a combination of a genetic programme that came about accidentally in evolution with mechanisms that involved self-organization. This will require integration of the historical paradigms of mathematical biology and molecular genetics [47]. It is in this integration that systems biology differs from both mathematical biology and molecular genetics, and in fact from mainstream physics and biology [49].

Systems biology also differs from physiology, which describes the functioning of biological systems in their entirety, without complete reference to the components. Cell physiology helps describe qualitatively how ATP levels change when muscle is innervated and why this leads to contraction. It does not explain this in a mode that predicts on the basis of changes in molecular processes.

19.1.2 What Systems Biology?

Systems biology has existed for more than 10 years now. Some of the low-hanging fruits have been picked. This included the discovery of interesting potential patterns of networking [2] and regulation [3] based on computational analyses of the completely sequenced genomes. However, even definitive information that two network components can interact does not certify that they actually do interact or that the flow of mass or information flux between the two components is significant. A transcription factor can interact with a gene only under the, possibly rare, condition where the former is actually expressed. A metabolite for which an enzyme has a binding site may only rarely attain concentrations that exceed its binding constant in the compartment the enzyme resides in. Without dynamic information about the actual states of the living systems, conclusions about scale-free intracellular networking and about prevalent gene–network motifs for biological function are preliminary.

Understanding of network function requires the experimental determination of the kinetic or binding properties of the macromolecular components. Systems biology should then assemble this information into a mathematical replica and calculate the fluxes. The latter should then correspond to what is measured experimentally. Lack of correspondence should be taken as a lead to discovery of new interactions or parameter values.

19.2 How Systems Biology?

Accepting the above ideal scenario for systems biology, one should translate this into something that is operational. At present this is almost impossible, because

too little is known or can be measured quantitatively. In addition, some parameter values are ‘soft’, i.e. depend on intracellular conditions that are not quite known. Examples are expression levels, and $V_{\max}$ and K_M values that depend on pH or even on the concentrations of other medium components [39]. In addition, it is difficult to measure the property of some enzymes, whereas it can be easier to do this for others. The strategies for systems biology have not yet been tried out. Below we shall review some such strategies, in particular the ones that relate to the silicon cell.

19.2.1 Top-Down Systems Biology

The strategy that is closest to genomics is called top-down systems biology [1]. Here the concentrations of all components of a certain class (mRNA, proteins, or metabolites) are measured in a genome-wide sense, as a function of time or of conditions. The components that behave similarly are then grouped together, assuming that correlation indicates a mechanistic or functional relationship. This may then lead to the proposal that all members of a group are regulated by the same transcription factor. Such a hypothesis may then be tested by identification of that transcription factor. It may also lead to the proposal of a temporal sequence of the action of regulatory molecules, hence to a regulatory pathway. Risks include the confounding of causes with effects, as well as the fact that regulation does not proceed through a single level of cellular organization (such as mRNA levels) but tends to involve at least gene expression and covalent modification through signal transduction, if not metabolism as well.

19.2.2 The Silicon Cell

The silicon cell approach [32, 41] is a strong form of the so-called bottom-up systems biology. The approach has been elaborated most for metabolic pathways. It consists of isolating all the enzymes of the pathway that is studied and of determining their kinetic properties, as well as their $V_{\max}$ s. The rate equations of all these enzymes are then put into a computer model, together with balance equations that give the change in time of the concentrations of all the metabolites as functions of all the reaction rates. The resulting system of equations is solved numerically for steady state or, after addition of initial conditions, for time evolution. Thus a computer replica of a biochemical pathway is created with behaviour identical to real behaviour, if the model is right.

The above approach may not seem new, but in its precise sense it is: although silicon cell type models have been made before, in many cases kinetic information was taken from databases for enzymes assayed under conditions that were not the same for all enzymes, nor corresponded to the condition *in vivo*. The silicon cell models of human erythrocyte glycolysis [27], of *Trypanosoma brucei* glycolysis

by Bakker et al. [5], of yeast glycolysis by Teusink et al. [36], and of the bacterial phosphotransferase system by Rohwer et al. [29] are early examples of what is close to the silicon cell approach. Yet, some of these were imperfect because the kinetics of the pathway enzymes were determined in cell extracts rather than with purified enzymes, or the cells were derived from fairly undefined pre-culture (which was, however, of immediate relevance for the application, e.g. baker's yeast).

The silicon cell is a rather loose research program that is greatly stimulated by the JWS Online modelling web site [33]. JWS (short for Java Web Simulation project) is a 'live' model repository, from which mathematical models of biochemical pathways can be downloaded in SBML form (Systems Biology Markup Language is the model specification language through which systems biology models are exchanged between modelling platforms [16]). The model repository is 'live' in the sense that the models can also be run through a web interface to JWS Online, without downloading. A user can therefore be completely ignorant of modelling and still do experiments *in silico*. The models come with the standard parameter set taken from their primary publication, which should correspond to the standard physiological state. Parameter values can be altered, and then the changes of concentrations and fluxes can be calculated as functions of time. In addition, systems properties such as the magnitudes and the control of steady-state fluxes and concentrations can be calculated. Before acceptance of models, JWS Online checks that they reproduce the simulations and calculations they express a claim to in their original publication. Because this reproduction is rarely complete, this model repository has an important function in quality control. BioModels, with which JWS collaborates, is another model repository with an even larger set of mathematical models, most of which can also be simulated in the JWS Online simulator via a direct link within BioModels. Its models have a more systematic annotation facility [23].

The way JWS Online is populated with models is not completely systematic yet, because there is too little funding for JWS Online *per se*. Consequently, the first generation of models in JWS Online were made by the small JWS silicon cell community. The second generation consists of models published in the high-quality scientific journals (e.g. *FEBS Journal*) that became interested in the quality control aspects of JWS Online. As part of their refereeing procedure, models in submitted papers are put into a (non-public) version of JWS, and the models are run to check that they produce every figure and table in the submitted manuscript. Perhaps surprisingly, this quality control mechanism finds faults with more than 90% of the submitted manuscripts. Only if the paper is accepted, the model becomes part of JWS Online (unless the authors do not want it to). In addition, there are a number of models that have been contributed to JWS Online by authors interested in getting their model used by colleagues through JWS Online or getting their citation numbers increased. It is the second and the third modes of contribution that should become most important in the future.

In Figs. 19.1, 19.3, and 19.5 of this chapter we give just three examples of silicon cell models. More such models are in JWS Online, e.g. accessible through <http://jjj.mib.ac.uk/index.html>. When going through the models in the JWS repository the reader will find some diversity. However, she/he will also recognize that

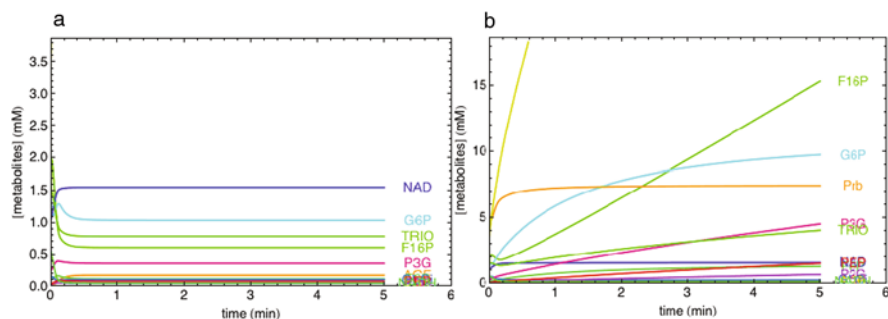


Fig. 19.1 Non-robustness of a silicon cell for yeast glycolysis. Development in time of a number of concentrations. **(a)** The normal state (see www.jjj.bio.vu.nl for the model [36]). **(b)** The same but after increasing the $V_{\max}$ of glucose uptake from 95 to 150; the concentrations of pyruvate and fructose biphosphate fail to reach steady state.

the variety of models is not representative for biology or even cell biology. This is because until now some parts of cell biology have led to more computer replica than others or because some authors have not submitted their models to JWS Online. Reason for the relative abundance of metabolic and especially glycolytic models is that in metabolism the law of conservation of the elements has direct consequences: at steady state, what flows into any node of the network must be equal to what flows out. This helps tremendously when defining the models and the associated experiments. This has led to such metabolic models being much more concrete and complete than signal transduction and gene expression models. Moreover, silicon cell models require accurate experimental data. Until recently, these were obtained either in extracts of cells or with enzymes purified from wild-type cells. In both cases, highly active enzymes are analysed most readily and hence the pathways that carry most flux can be approached most successfully.

Most models in JWS Online are of the bag-of-enzymes type, i.e. they assume that enzymes convert metabolites that are present in well-defined pools and that there is no direct transfer of metabolites between enzymes, i.e. no metabolite channelling. Likewise, enzyme sequestration by binding to other macromolecules, macromolecular crowding, and active structuring are underrepresented, as are metabolic pathways that are subject to adaptation through gene expression regulation. These issues are underrepresented, but they are not absent. In particular the silicon cell model of the *Escherichia coli* phosphotransferase system [29] is rich in these complications: it addresses signal transduction, transport, channelling, and macromolecular crowding. Gene expression regulation and DNA structure-regulating gene expression are modelled in [34]. Many models are about steady states and the approaches to steady states. However, in biology systems with steady states as the main attractor dominate and the rather large numbers of models in JWS Online that deal with oscillations may actually over-represent oscillatory systems. They include yeast glycolytic oscillations (e.g. [50]), the cell cycle [9], and oscillations in NF κ B signalling [17].

19.2.3 *Silicon Cell Models: Advantages and Disadvantages*

What is the advantage of having a silicon cell type model, a ‘computer replica’, of a biochemical pathway? If perfect, such a model is just as complex as reality; hence it does not correspond to the abstraction and simplification of reality that is often associated with ‘understanding’. Mathematical biology has long made models of biological systems that aimed at this type of ‘understanding’. Why not stay with those models of mathematical biology?

Examples of such mathematical biology models include the Turing type of models which were used to show that self-organization might explain pattern formation in developmental biology [51, 52]. These models each contained a simple network with positive and negative feedbacks. Using simple parameter values their predictions were calculated and shown to lead to pattern formation. Most often no attempt was made to produce a precise correspondence between simulation results and experimental data. Where such attempts were made and the predictions of the model did not fit experimental observations, the parameters in the model would be adjusted until a statistically satisfactory fit was obtained. In principle the fitted parameter values could then be verified experimentally, but this is rarely undertaken in practice: the number of parameters exceeds the number that could be determined experimentally at the required level of accuracy, or, more often, the parameters refer to abstract properties that cannot be measured directly. Even if a parameter value could be measured and was shown not to correspond to what was assumed in the model, then other parameter values would be adjusted so as to obtain a renewed fit between model prediction and experimental system behaviour. Only if such fitting would prove completely impossible the model could serve the important function of falsifying a hypothesis about mechanism, but this has been rare. More often, parameter values could be found for which the model fitted the experimental behaviour, but there was no assurance that those parameter values corresponded to reality. For instance the model would fit the data if a lower than actual $V_{\max}$ was inserted for an enzyme (such as hexokinase in [37]). The fitted model would be wrong mechanistically, even though it would appear to explain the phenomenon of interest, such as pattern formation.

The resulting model could still be used, but then as a phenomenological, descriptive model. Phenomenological models have a long and successful history in both physics and engineering. In physics, because of greater simplicity, subsequent experimental testing was possible and often led to reformulation in terms of a more detailed, mechanistic model and then validation or falsification. In engineering the models were considered useful also without such validation, because the purpose of a model was the description of the behaviour of the system, not necessarily an explanation of how that behaviour was actually achieved. Most of biology is different, however; it is much more complex than physics, actual detail matters (see above), and it often wishes to relate physiological behaviour of the system to its components’ properties. The latter is important for metabolic engineering and therapeutic purposes.

Now we get to the answer to the question why one could not stay with the usual models of mathematical biology. The reason is that they do not enable one

to validate that proposed mechanisms are actually operative in, and explanatory for, observed functional behaviour. Silicon cell models are realistic and suitable for a falsification/validation strategy. This is a prime utility of silicon cell type of models, i.e. scientific validation/falsification of proposed understanding of systems.

Although silicon cell models do not themselves constitute understanding in the sense of simplification to what is most important, they do instantiate another type of understanding, i.e. that of the ability to predict. If the prediction fails to correspond to reality, experimental follow-up can lead to improved understanding. In other words, silicon cells are the tools that are ultimately required for the continued development of our understanding of biological systems.

In addition, silicon cell models can contribute considerably to understanding by enabling computational experiments. Complex actual mechanisms may be elucidated more readily by interrogating a computer replica of reality through computational biology, than by experimental biology. Figure 19.1 illustrates how this has worked already. It shows that the silicon cell model of yeast glycolysis was rather unrobust with respect to the activity of the glucose import system; as shown in Fig. 19.1b only a slight increase in that activity could lead to a 'metabolic explosion', i.e. to a continued increase in the concentrations of some metabolites. Because real yeast is robust in this respect, but a mutant is not, this led us to understand an aspect of the 'turbo' organization of many catabolic pathways that could lead to fragility and then to a hypothesis on how a regulatory interaction for which no function was known and which had not been included in the silicon cell, might be quite important for yeast glycolysis [37].

Silicon cell models have two additional advantages. One is that their parameters are 'hard' in the sense that they correspond to properties of real molecules. This means that, once known, the parameter values should not change anymore unless the model is wrong or the properties of the molecules involved change. Fitted, phenomenological models have the disadvantage that for every new experiment the entire model should be refitted to all existing experiments, allowing all parameter values to be adjusted so as to make the fit optimal [25]. For large models this can become increasingly bothersome. The second additional advantage of silicon cell models is that because they are formulated in terms of real entities, models that address adjacent parts of cell function tend to be formulated in the same terms or in terms that can be readily translated into one another. Thereby, the silicon cell strategy should allow for the assembly of some of its models into larger models. Related to this, the silicon cell initiative furthers standardization. Many modellers like to see their models used by others in a wider context and are therefore willing to standardize them. The development of SBML [16] is a sign of this, but the silicon cell initiative tends to go further in certain aspects. Whereas SBML is a standardization of a model description format, we aim for a standardization of model construction protocols.

The silicon cell strategy also has many disadvantages. One is that it requires an awful lot of careful experimentation to determine all the kinetic parameters. In addition it requires *all* components to be assayed, which is impossible for realistic systems, first because they contain too many components and second because there is always a component that is most difficult to isolate or assay.

Information and Complexity

The works of Claude Shannon (1916–2001) and Andrei Kolmogorov (1903–1987) made the terms *quantity of information* and *complexity* important theoretical notions for the second half of the twentieth century. Roughly speaking, the quantity of information is simply measured by the minimal length of a binary-coded string of signs representing the given information. Today, the concept is commonplace in loss-free compression of (large) data files for archival storage and transmission over phone lines. ZIP programs are ubiquitous, typical hybrids of ideas going back to David A. Huffman (1952), radically modified by Abraham Lempel and Jacob Ziv (1978), and further modified jointly with Terry Welch (1984).

This purely quantitative concept of information quantity can be disorienting: it must be preposterous to measure the information content of Edgar Allan Poe's *The Raven* by its sheer number of lines. Similarly, in cell biology we need to know whether information is important and reliable: these are qualitative rather than quantitative characteristics.

What then is the use of a purely quantitative aspect of information, not bothering about its meaning or reliability – like in modern media industry when it is worst? Elaborating on its undoubted reasonable application in data storage and transmission technology, we can consider the task of loss-free compression as encoding a program P at the source for obtaining the decoded text Q at the target. The length of the shortest programs like this is well defined and called the *Kolmogorov complexity* of Q . Consequently, the Kolmogorov complexity of the first billion decimals of Euler's number $e = 2.718\dots$ is very small due to the simplicity of the algorithm $e = \lim_{n \rightarrow \infty} \left(1 + \frac{1}{n}\right)^n$ which yields e with any wanted precision. Similarly, graphs of seemingly immense complexity like the Mandelbrot set in complex dynamics have extremely low complexity. The same is true for a modern physical theory where a rich world of phenomena is generated by the handful of relatively simple laws of classical mechanics, electromagnetism, thermodynamics, relativity, and quantum mechanics. These laws yield principally unlimited precision for as long as one sticks to some restricted variety of stuff: massive point particles obeying only the law of gravity; electromagnetic field in a vacuum; and the like. In spite of these limitations, ideally simple physical theories can be very useful in engineering, e.g. for designing a machine as an “artificial fragment of the universe where only a few physical laws are allowed to dominate in a well isolated material environment” (Y. Manin).

For β -cell functions, a comparable simplicity, low quantity of information, and low Kolmogorov complexity are hardly achievable, as foreshadowed by the saying attributed to Einstein: “Everything should be made as simple as possible, but no simpler.”

Further Reading:

Cover TM, Thomas JA (1991) *Elements of information theory*. Wiley, New York, NY

Manin YI (2007) *Mathematics as metaphor* – with Foreword by F.J. Dyson. American Mathematical Society, Providence RI

Added by the editors

A second disadvantage is that it is excruciatingly slow and not always maximally exciting. For instance, the silicon cell approach suggests that having made such a model for an organism for a particular experimental condition, one should start all over again if one is interested in a different organism or a different condition; the

organism may then express different isoenzymes. However, repeating the procedure for the different condition, one may obtain the same result in terms of true understanding of function, as one had obtained for the original conditions and organism. On the other hand, quite similar organisms may have entirely different functions or mechanisms which they may achieve by differences in networking of essentially the same molecules (compare [13] to [37]). This issue now leads to comparative systems biology.

A third disadvantage is that until now, the actual silicon cell models have been about parts of cell function that were considered to belong together, such as metabolic pathways in their classical definition. Strategies for a more rational definition of what pathways silicon cell models should begin to focus on, are being developed [49].

19.2.4 Blueprint Modelling

Blueprint modelling tries to deal with this demotivating feature of having to redo silicon cell models of related organisms and with the motivating feature of comparative systems biology. The blueprint procedure starts from the silicon cell model that is already available of a related organism and then changes this in the light of what is already known of the molecular properties of the organism under study. Comparing the predictions of this adjusted blueprint model with physiological behaviour measured experimentally, one then prioritizes which parts of the blueprint model need to be detailed further.

19.2.5 The Wisdom of MOSES: Domino Systems Biology

Intracellular networks are vast and virtually completely connected. In principle, a true silicon cell model is a model of the total expressed genome. This is impossible to achieve, at least for the foreseeable time, and one needs to start with a part of the intracellular network. Ways to divide the intracellular network into modules that can be considered separately are highly important therefore [15, 30, 31, 38].

‘Domino systems biology’ begins at a key metabolite and then uses pre-existing knowledge concerning the pathways and processes that synthesize this metabolite and the processes that consume it. It determines, by using pre-existing pathway models from silicon cell, by performing new in vitro enzyme kinetic assays, or by modular kinetic analysis [8], how these processes depend on the concentration of the key metabolite. Starting with the most important synthesis process and the most important degradation process, it then formulates a first model with the intermediate in the middle and the two processes around it. It then predicts how activation of the processes affects the concentration of the intermediate at steady state and the fluxes and compares this with the results of corresponding experiments. Failure of the model to predict the latter type of observations is then used to invoke either an

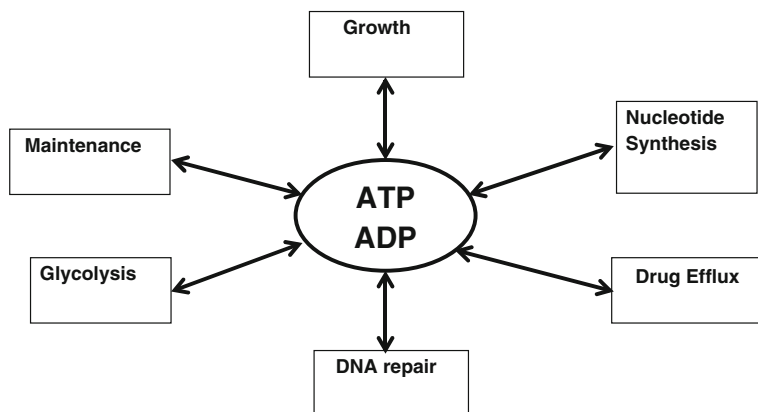


Fig. 19.2 Several modules linked by their consumption, production, or other interactions (e.g. allosteric) with the adenine nucleotide pool

additional process or an additional metabolic intermediate. By incorporating a next additional process or metabolite one adds the next domino stone.

Micro-Organism Systems biology: Energy and *Saccharomyces cerevisiae* (MOSES) is a research program that develops domino systems biology for yeast. Figure 19.2 shows the example for when one takes ATP as the central intermediate, which is relevant for cellular energetics. Figure 19.3 shows a modelling result that comes from this approach, i.e. a perhaps somewhat paradoxical dynamic behaviour of the ATP level upon activation of the glycolytic pathway producing ATP [35].

19.2.6 Metabolic Control Analysis Models

Another strategy to enable precise modelling does not seek to limit the network size, but to reduce the types of questions that are addressed by the model. Metabolic control analysis is such an approach. It only addresses the control of fluxes and concentrations, not their magnitudes. It is possible to calculate the flux and concentration control coefficients from enzyme kinetic properties called elasticity coefficients [18, 28, 44, 45]. Elasticity coefficients contain limited information about the enzymes that participate in the pathway and can hence be estimated in the absence of the full information. Galazzo and Bailey pioneered this approach experimentally using a fair number of rather precise rate equations which enabled them to calculate the elasticity coefficients, because they had measured the intracellular concentrations of some metabolites by NMR (nuclear magnetic resonance) [12]. They found much but non-exclusive control of the flux by the glucose transport system, but this was partly the result of a proposed inhibition of the transporter by glucose-6-phosphate, for which there is no direct experimental evidence.

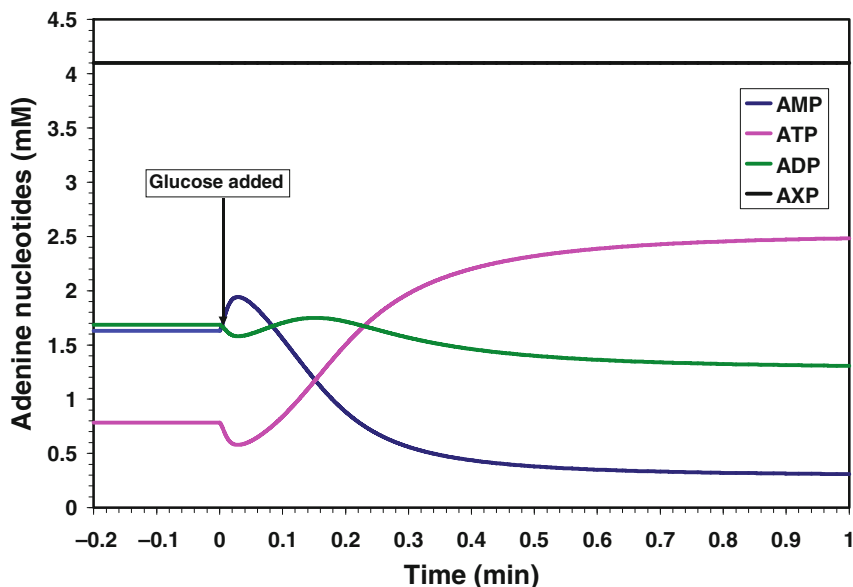


Fig. 19.3 Adenine nucleotide dynamics for glucose perturbation by integration of glycolysis and maintenance modules. $AXP = ATP + ADP + AMP$.

19.2.7 The Silicon Cell Strategy in Yeast

Of course an alternative to the above approximate approaches is to carry out the silicon cell agenda as completely as possible. It is indeed one of the main aims of the Manchester Centre for Integrative Systems Biology to provide a first, fully predictive, and essentially complete systems biology of the most important function of an organism, in terms of a silicon cell model. The initial strategy was to over-express and partially purify each enzyme of yeast and then to determine its kinetic and interactive properties. This approach was not efficient enough, as high-throughput kinetic assays were only successful for some enzymes. For most others the substrates were not available commercially or the enzymes were too unstable.

Therefore it was decided to leave this genomics-driven strategy and to switch to a function-driven strategy, i.e. to select a function of interest, estimate which enzymes are most involved in that function, isolate and characterize those enzymes, and then make a silicon cell model [49]. The resulting strategy is illustrated in Fig. 19.4.

19.2.8 Silicon Cell and Differential Network-Based Drug Design

Most drugs have multiple effects on the patient. One reason is that their targets are parts of molecular networks that connect with other networks. The concept that drugs should be targeted at single molecules may be good for the ability to

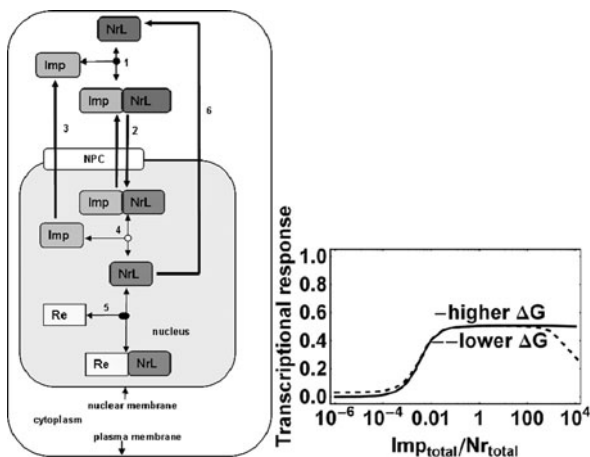
19.2.9 The True Silicon Cell

Until now the words ‘silicon cell’ have been misnomers. All that exists presently, as exemplified by the collection of models on the JWS Online model repository, are models of, mostly metabolic, pathways. There is no model of an entire cell. The name silicon cell stems from the ambition ultimately to combine silicon cell models of pathways into models of entire cells.

Cells are compartmentalized and involve more than metabolic pathways. Figure 19.5a shows a network that is not involved in metabolism but in signalling. It represents a blueprint model of nuclear hormone receptor signalling in various human cell types. Nuclear receptors (NRs) belong to a family of transcription factors involved in a diverse range of regulatory functions, such as the ones that are active during development, inflammation, and metabolism [7]. An NR is a protein that is synthesized in the cytoplasm, shuttles between the nucleus and the cytoplasm, and binds with its response element on the DNA. Addition of ligand L results in the appearance of NRL in the cytoplasm. Then, NRL shifts into the nucleus and binds to response element, causing a transcriptional response (Fig. 19.5b).

A curious aspect is that both export of importins and export of liganded receptor are driven by RanGTP hydrolysis. Why would the cell spend free energy on these processes that both seem to work in the wrong direction? We made a model of this network which is on its way to but not yet equivalent to a silicon cell model; many kinetic parameters are still unknown. In this model we asked what would happen if we decreased ΔG (i.e. the Gibbs free energy difference) of both processes by a factor of 100 (dashed line). We found that the high investment of Gibbs free energy would stimulate transcription at high concentration ratios of importin to nuclear hormone receptor. This leads us to formulate the hypothesis that the investment of free energy serves to prevent sequestration of nuclear receptor by importin.

Fig. 19.5 Silicon cell model of a nuclear hormone receptor signalling network (a) and prediction (b) of the dependence of transcription activation on the total concentration of importin (Imp) in the system and the free energy driving the transport cycles



19.2.10 Crossing the Scales

In the above venture from pathway to cell, we met the complication of an extra compartment. When two compartments have different volumes, processes in one compartment are likely to have different kinetics from processes in the other. Even in a single compartment, time scales may be diverse. One origin of this is in the gene expression cascade. The concentrations of the enzymes in metabolic pathways may adjust to changes at the level of metabolism through regulated gene expression. Because the lifetimes of proteins are mostly longer than the lifetimes of many intermediary metabolites, the dynamics of gene expression are often quite a bit slower than the dynamics of metabolic changes. The methodology of rate and balance equations that has mostly been used in the silicon cell up to now can deal with a full range of dynamics. However, for conceptual purposes, and for the purpose of more rapid computation, methods that summarize the behaviour of more detailed, faster scales into behaviour at the often more relevant, slower and less detailed scales are important [11].

A well-known issue at the faster time scales is that of the dynamic behaviour of enzymes. This can be described at the level of the free substrate [S], the free enzyme [E], and the enzyme–substrate complex [ES] or at the level of the total substrate and the total enzyme concentration. The latter has the advantage that total enzyme is set by the dimension of gene expression and not by metabolism. Even though the quasi-steady-state approach (QSSA) to enzyme kinetics is a fair way to deal with this usually, there are recent methods for dealing with the cases where enzyme and substrate concentrations are comparable and the QSSA fails [11, 14]. Metabolic control analysis (MCA) has been extended to address the multi-scale issue of signal transduction [19] and of that metabolism versus gene expression [42, 46], also experimentally [14, 34].

When moving up from the cell level, to the whole body, additional scales appear, such as the scale of the circulation, which is important for the organism action of β -cells. The coupling of models of the silicon cell type should again help at those scales. We shall discuss this below.

19.2.11 Different Types of Modelling

This chapter is motivated by the question of how molecular issues in β -cells might be put in the perspective of their biological function. Since their biological function is at the level of the whole human, this involves the crossing of temporal and spatial scales from molecules to the whole mammalian body. Apart from, but sometimes related to, the scales at which one is considering these issues, there are different modelling methodologies. Above we have discussed a few, i.e. top-down systems biology, blueprint modelling, domino systems biology, metabolic control analysis, and silicon cell. Three of these five modelling methodologies involve balance equations and kinetic equations. Metabolic control analysis uses less than this [28, 44],

but is limited to control aspects. Top-down systems biology tends to lead to phenomenological models describing patterns. There are quite a few other modelling methodologies that we have not discussed until now. This is because this chapter is devoted to describing the methods that we find most important for obtaining a useful mathematical representation of the human that enables to relate her function to her molecules.

This is not to say that other modelling methods are not more useful for other important problems, or even that they will not be important for some aspects of the silicon human. For instance, flux balance analysis as a modelling method may help establish where to look first for important pathways [45]. However, it ultimately suffers from the fact that we do not know what the relevant objective functions are. A sole objective function of maximum yield of ATP is likely to be irrelevant for most human cells.

Modelling cells in terms of Boolean networks may be quite helpful for initial understanding, but suffers from the limitation that in reality the intracellular networks are based on ensembles of molecules and not on individual chains of molecules. Therefore only after it has been shown that parts of the intracellular networks do indeed act as switches, one could engage this method. Transcription does not yield a single mRNA molecule that is then translated into a single enzyme which then makes a single molecule of the product. The difference matters for life, which critically depends on the ability to deal with the challenges imposed by the second law of thermodynamics. The latter depends on the laws of larger numbers and entropy [48]. Boolean networks have no problem with violating the second law of thermodynamics. Bayesian networks are a more subtle alternative to Boolean networks, allowing for event probabilities in between 0 and 1. Living systems operate in states that are steady or steady on average. Thereby part of the essence is not how they move from one state to the next, but how a single state is functioning. For sure, when a glucose molecule enters a tumour cell, its C1 carbon atom has a certain probability to end up in carbon dioxide and a different probability to end up in lactate. One would be interested in how these probabilities are influenced by the expression of glycolytic genes. This is a matter of a steady-state balance between rates, the implications of which for metabolite concentrations are modelled best by rate and balance equations. Bayesian networks operate by forward logics, i.e. what happens can only be determined by the present and not by the future. Already shortly after activation of intracellular networks, what happens in their beginning is co-determined by what has happened at their end. At steady state the end of the pathway just coexists with its beginning: the former depends on feedback loops through the latter, one of the reasons why the first step is not completely rate limiting. Bayesian networks do not seem to accommodate this essential, feedback property of living cells.

One interpretation of ‘computer replica’ of the living organism would indeed model the system in terms of all its individual molecules as they are interacting. This would inspire a gigantic Monte Carlo simulation including the quasi-Brownian motion through Cartesian and chemical space. This, however, would generate models that are more complex than can be calculated in the lifetime of the planet, even

after introducing the simplifications offered by biological organization discussed above. In addition it would depend on the initial conditions of all the individual molecules, which one could never determine. It would also be impossible to trace the behaviour of every individual molecule, without perturbing it; this problem is not unique to quantum mechanics. The silicon cell project models mostly in terms of ensemble-averaged concentrations whenever this is feasible on the basis of statistical mechanical considerations [48]. Stochastic modelling does become important when molecule numbers in the relevant compartments are below 100. This is rare, though occasionally important. Partial differential equation based modelling is needed when gradients within compartments become important [20]. Biology has become systems biology and hence realistic science. It has to make its information and analyses as simple as realistic but not simpler (free after Einstein; see the box).

19.3 Towards the Silicon Human

In the context of the human, the ambition is even greater, i.e. to combine models of cells into models of tissues and then to combine models of tissues into body-wide models. Because the cell models would still be in terms of molecular activities, the result would be a multi-scale model relating whole-body function to molecular activities in time and space. Here, the silicon cell project will become a silicon organism project, with variations such as the virtual physiological human and the digital human projects. The idea is similar to that of integrating pathway models. Relatively autonomous models of organs are to be combined. One thought is to leave the coordination of each organ model including the corresponding computations to an individual research centre and then to integrate the models dynamically through web services. Although perhaps slow, this would have the advantage of maximum responsibility of a group over a part of the whole model, ensuring quality control. Figure 19.6 illustrates this approach, where of course the β -cell component model will play an important role. Another thought has models for parts of the system uploaded to JWS Online by the respective research groups, these models being automatically merged into the complete model, available to all participating groups.

Pharmacokinetics has already studied the human body as a multi-compartment problem. Recently it has been proposed that more mechanistic information should be incorporated into pharmacokinetics, moving the subject to an integration of pharmacokinetics and systems biology [21]. We are therefore elaborating the silicon cell approach for tissue–tissue interaction in the whole human body. We thereby focus on the part of Fig. 19.6 that is depicted in Fig. 19.7a. The pancreatic β -cells, shown schematically on the left, are connected with a model for C-peptide kinetics. Based on experimentally measured C-peptide levels in a patient we are able, using this model, to estimate the dynamic and static components of the insulin secretion, the former being a function of the glucose concentration above a certain threshold level and the latter being a function of the rate of increase of the glucose concentration.

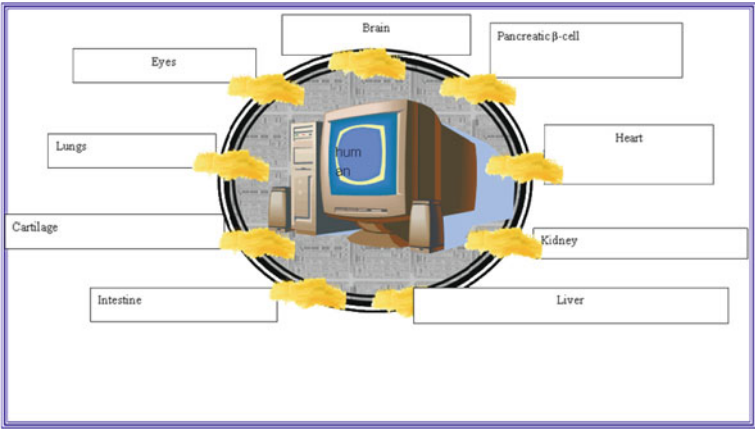


Fig. 19.6 The silicon human as a collection of dynamic models of its organs

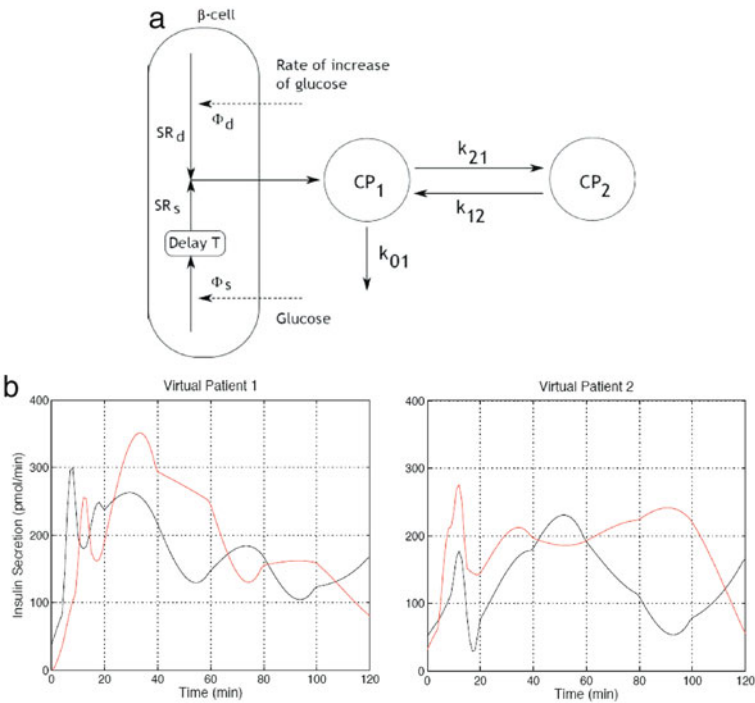


Fig. 19.7 Minimalistic whole-body silicon cell model relevant for insulin, glucose, and C-peptide dynamics and some of its predictions. **(a)** The scheme referring to the insulin release model and C-peptide kinetics. **(b)** Calculations of insulin secretion after administration of glucose for a silicon human subject to a normal (the line that is the highest in the beginning) and a hypercaloric diet. **(c)** The same calculations for a different silicon human.

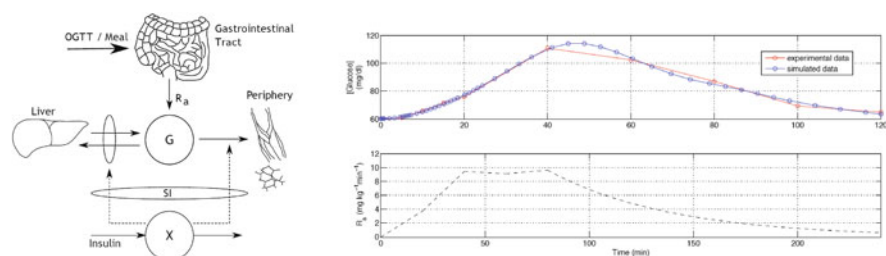


Fig. 19.8 Another minimal whole-body silicon cell model relevant for insulin and glucose dynamics and some of its predictions. (a) The scheme referring to interplay between insulin and its effect on glucose utilization and storage. (b) Calculations of glucose absorption profile during an oral glucose tolerance test (*bottom plot*) and fitted glucose time course (*top plot*).

Figure 19.7b and c gives the results of calculations for two different silicon humans (i.e. different mechanistic parameter values for the two models) of insulin secretion rates in the normal and in the hypercaloric states. Figure 19.8a illustrates a complementary model for glucose and insulin dynamics. It allows for estimation of the insulin sensitivity of a virtual patient, a numerically calculated measure quantifying the interplay between insulin level and the ability of the organism to balance its glucose concentration. The figure shows that, provided individuals can be characterized in terms of a few mechanistic parameter values, implications of food intake for insulin dynamics can be predicted. At this stage, it is unclear whether those predictions would be correct or not, but this is now accessible to experimental validation.

To many, the idea of a silicon human seems too complex to even think about. This may, however, derive from a failure to appreciate that biological organization greatly reduces complexity [43]. Moreover, the silicon human is already developing. Models of important aspects of the heart [24] and of the liver cell [40] are constructed. Thirty years from now we will avail of thousands of mathematical models that each describe a part of the human. Perhaps the only strategic decision we need to make now is whether all those models will have resulted from a cottage industry such that it will be impossible to integrate them with each other, or all those models will have been developed in a common context and can be merged into a larger, more complete model. The latter possibility should enable each researcher working on her/his part of the human to appreciate the implications of her/his findings for understanding the functioning of the human as a whole. And, because there will be simultaneous top-down and ‘middle-out’ [24] strategies towards mathematical models of the human, we also have another choice. Either the results of these three methodologies will be developed independently of each other and the results will be in different languages, or some time is spent now to ensure that ultimately they become continuous with each other. The choice is (y)ours.

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Chapter 20

Probing Cellular Dynamics with Mesoscopic Simulations

Julian Shillcock

Abstract Cellular processes span a huge range of length and time scales from the molecular to the near macroscopic. Understanding how effects on one scale influence, and are themselves influenced by, those on lower and higher scales is a critical issue for the construction of models in systems biology. Advances in computing hardware and software now allow explicit simulation of some aspects of cellular dynamics close to the molecular scale. Vesicle fusion is one example of such a process. Experiments, however, typically probe cellular behaviour from the molecular scale up to microns. Standard particle-based simulation techniques cannot capture such a broad range. Consequently, at long length scales, models have often been of the mass action variety, in which molecular constituents are represented by density fields that vary continuously in space and time, rather than involving discrete molecules. But these models struggle to represent processes that are localized in space and time or involve the transport of material through a crowded environment. A novel class of mesoscopic simulation techniques are now able to span length and time scales from nanometres to microns for hundreds of microseconds and may soon be coupled to mass action models allowing the parameters in such models to be continuously tuned according to the finer resolution simulation. This will help realize the goal of a computational cellular simulation that is able to capture the dynamics of membrane-associated processes such as endo- and exocytosis.

Keywords Computer simulation · Mesoscopic model · Multiscale modelling · Dissipative Particle Dynamics · Endocytosis · Exocytosis · Vesicle fusion

20.1 Introduction

Biological cells appear in many shapes and guises but share a lot of structure and machinery. Common to all mammalian cells, the plasma membrane (PM), which

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encloses the cellular cytoplasm, is composed of hundreds of different types of lipids and proteins. Lipids are amphiphilic molecules that have a hydrophilic (water-loving) head group bound to one or more hydrophobic (water-hating) tails. Because the tail segments do not want to be immersed in water, lipids in solution tend to self-assemble into structures that segregate the tails away from the surrounding water. These structures can take several forms, of which the most common are spherical droplets of lipid, called micelles, and closed sacs of water surrounded by a double layer (or bilayer) of lipids called vesicles. In micelles, the lipid heads point out into the water phase while their tails are shielded in the core behind the heads. The vesicle bilayer consists of two adjacent layers of lipids arranged so that their heads point outwards from the layer and their tails are sequestered between the two layers of head groups. The PM of cells consists largely of a lipid bilayer in which proteins are embedded, and the lipid and protein molecules are free to diffuse around.

The PM not only forms a protective casing around the cell but also controls the transport of material into and out of the cell and functions as a signal transducer so that the cell can sense, and respond to, its external environment. Idealized models of the PM have been constructed since the early 1970s, when Singer and Nicolson [37] proposed their “fluid mosaic” model in which the lipids form a planar “sea” within which membrane proteins float around performing their functions. This model has recently received further scrutiny [7] that has indicated that the proteins are actually rather crowded instead of being widely separated on a sea of lipids. But it still forms the basis for most conceptual understanding of the PM.

The cell has to perform dynamic modifications to the PM during its lifetime. Some of these relate to cell division or the endocytosis of nutrients and exocytosis of materials such as hormones. During exocytosis, small vesicles or granules, which have diameters in the range 50–100 nm, are moved to the inside of the PM, fuse with it and release their contents. This process of fusion and content release is the primary function of β -cells that produce insulin for use in controlling carbohydrate metabolism. The production and release of insulin by β -cells are highly complex and cannot be observed directly using light microscopy as the granules are too small. However, it is clear that the following steps must occur before and during insulin release: insulin granules are packaged with their cargo of insulin; the granules are moved towards the PM where they are held in a form of holding pattern until needed; upon receipt of the appropriate signal, granules are driven to fuse with the PM and release their contents to the extracellular space.

Endo- and exocytosis take place on length and time scales from nanometres to microns and nanoseconds to milliseconds and longer. This range of length and time scales that are larger than molecular scales but not quite macroscopic has been called the *mesoscopic* realm (meso = middle in Greek). Experimentally, it is difficult to probe dynamic processes on the mesoscale because a typical experiment integrates over a macroscopic time period of many microseconds or longer, during which molecules can undergo massive reorganization. Electron microscopy can reveal atomic details but only offers static snapshots of the events of interest. Conversely, theoretical models of the PM tend to be mathematically idealized

continuum models, such as elastic sheet models based on the Canham–Helfrich Hamiltonian [5, 15], that cannot predict the remodelling of the membrane that is inherent in topology-changing processes such as endocytosis or vesicle fusion. Theoretical models of membrane fusion assume a certain pathway for the process and calculate the free energy changes along this pathway [6]. It is highly desirable to have a method of probing models of the cellular PM in the mesoscopic realm that does not require a priori assumptions. This would allow the testing of hypotheses about dynamical phenomena in the cell and their possible alteration in disease states. In the specific context of β -cells, the motion of the granules, their fusion and release kinetics, and the effects of changing their material properties, and those of their environment, on the cell's insulin release could be probed using the simulation. Particle-based computer simulations are beginning to capture sufficient detail about the cell's internal state that they can reproduce these crucial events. Soon, these simulations will be able to contribute to the iterative process of model-driven experimentation that is at the heart of systems biology (see [Section 1.4](#)).

20.2 Particle-Based Computer Simulations in Biophysics

Computer simulations have been used for more than half a century to study the behaviour of idealized physical systems. A simulation may be defined as the evolution of a set of entities that interact according to prescribed rules and the measurement of properties of the set that can be compared with experimental data or other theoretical models. The earliest simulation techniques that used explicit particles to represent atoms or molecules were molecular dynamics [29] and Monte Carlo [4] simulations. Both of these methods have been used to simulate molecules of biological interest such as proteins and lipids. Their usefulness stems from their minimal need for a priori assumptions about a system's behaviour. Once the molecular composition of a system and the intermolecular forces have been specified, the simulation yields unbiased information about the system's evolution. The ability to generate unexpected effects makes simulation a valuable tool in exploring the properties of complex dynamical systems. In this section, we give a brief overview of these simulation techniques and show why their limitations have led to the development of novel, mesoscale simulation algorithms that can capture phenomena beyond their reach.

Molecular dynamics (MD) simulations solve Newton's laws of motion for a collection of particles interacting by specified force laws within a given volume of space. The particles may be point particles (e.g. atoms) or extended objects (e.g. a whole lipid or protein) whose shape is defined by the interaction laws. Each particle has a mass, position and velocity, and the force on each particle is the sum of its interactions with all other particles. The set of forces that govern the particle–particle interactions is usually chosen so as to represent the actual physical forces acting in the real system. For a collection of molecules these would include van der Waals forces, molecular bond forces, electrostatic forces. Once the forces have

been specified, the Newtonian equations of motion of all the particles are integrated using an accurate algorithm [2]. The simulation then predicts the motion of the particles over time, and the system's evolution is related to the corresponding experimental system by taking averages of observables that correspond to quantities that can be measured in the experiments. For example, the temperature of a set of particles moving in a closed box is just the average kinetic energy of all the particles.

Monte Carlo (MC) simulations take a different approach. Instead of calculating the evolution of particles in time and constructing observables from the particle trajectories, the system of interest is placed in a specified thermodynamic ensemble (constant particle number, volume and temperature for example, the so-called NVT ensemble), and its microscopic degrees of freedom are modified according to a set of rules that take the system from one microstate in its ensemble to another. As long as the rules obey a condition known as detailed balance, one is free to choose rules that may or may not be physically realizable. The most common scheme for generating transitions between states of a system is known as the Metropolis algorithm after one of its originators [24]. The algorithm not only tends to drive the system towards its free energy minimum but also allows small deviations that are thermally activated. After an initial transient, the system reaches equilibrium and randomly samples states in the defined ensemble. The connection to experiment in this case is made by averaging observables over the sequence of states generated by the algorithm. Because the system as a whole moves through its phase space instead of the individual entities composing the system moving in real space, the sequence of states generated is not necessarily temporally related: it is often the case that only equilibrium states can be compared to experimental data.

In principle, MD and MC computer simulations could be used to construct dynamical models of the PM that are evolved in time and their behaviour observed under appropriate conditions [22]. But they both have limitations that prevent them being used for the kind of problems in cellular dynamics in which we are interested here. The traditional molecular dynamics algorithm requires calculating the forces between all atoms in the system of interest and has to use a small integration time step (of the order of 10 fs) because of the strong inter-atomic forces involved. The huge computational demand of such a calculation currently restricts the use of atomistic MD to simulations of single proteins and/or patches of membrane that are roughly 20 nm in linear dimension. They cannot therefore be used for exocytosis that occurs on 100-nm length scales. Monte Carlo algorithms can represent larger spatial systems, including membranes [11], but lack a realistic dynamics. Although this lack can be circumvented in special cases, it is non-trivial to choose a set of moves for the MC algorithm that allow one to observe the detailed molecular rearrangements of, for example, biological lipids in vesicle fusion. MC simulations are typically used to simulate polymeric systems because there is a large body of theoretical work with which the simulations can be compared.

The limitations of traditional MD and MC simulations have driven the invention of coarse-grained (CG) simulation techniques [3] that attempt to capture just

enough of the molecular details of the membrane's constituents (lipid and protein molecules) to reproduce its material properties and behaviour without demanding huge computational resources. Several recent reviews have dealt extensively with methods for simulating membranes [22, 25, 36] and provide an excellent entry to the field for readers wishing to examine the broad range of techniques available, some of which are still being actively developed.

Coarse-graining, a molecular system, involves averaging over certain molecular properties, or degrees of freedom, such as bond vibrations or hydrogen atoms, and replacing their explicit representation in the simulation by lumped parameters. A typical example is the coarse-graining of a polymer chain or lipid hydrocarbon tail. Instead of simulating each carbon atom (together with its bound hydrogen atoms) in a lipid tail, successive methyl groups are lumped into hydrophobic *particles* connected by Hookean springs. Each particle represents several methyl groups, and the parameters that control the simulated chain's length fluctuations (the spring constant and unstretched length) are chosen so that it exhibits the same end-to-end fluctuations as the polymer or lipid tail that it is meant to represent. Because the process of choosing which features to represent in CG simulations is something of an art, various coarse-grained simulation techniques have been created, which are optimized for different aspects of membrane behaviour. They all tend to have a rather large number of parameters which must be given appropriate values if the results of the simulation are to match those of the equivalent experimental system. How to calibrate the values of these parameters is the central task in setting up any CG simulation [26]. Ideally, this would be automated using either explicit experimental data or finer resolution MD simulations, but this is currently only possible in very few cases [26, 30].

One approach to coarse-graining that we mention in passing is to discard the explicit representation of the solvent (water) entirely and take its effects into account through more complex force fields (as in the ESPRESSO code, www.espresso.mpg.de), so-called solvent-free models, or by the assumption that the solvent simply produces stochastic fluctuations of the retained particles' velocities. The former method has been used to simulate large-scale membrane invagination [31]. The latter approach, termed Brownian dynamics, is a whole sub-field of simulations [2] and is particularly useful when the solvent is simply a uniform medium in which larger particles diffuse independently. It has been used to follow the self-assembly of hundreds of actin monomers into a filament, for example [14], and to study the fusion of small vesicles [27]. More information about solvent-free models can be found in two recent review articles [3, 36]. A final concern with constructing membrane models is to decide how much of the cytoplasm and extracellular environment to include in the model. Too little, and the model cannot respond appropriately to external changes, but including too much increases the computational demands beyond what is feasible. In the next section, we describe an increasingly popular method of coarse-graining a lipid bilayer membrane that has been found useful for simulating exocytosis: dissipative particle dynamics.

Buffon's Needle Problem – An Early Forerunner of Monte Carlo Simulation

The French naturalist Georges-Louis Leclerc, Comte de Buffon (1707–1788) is best remembered among scientists for his *Histoire naturelle, générale et particulière* (1749–1788 in 36 volumes), covering the animal and mineral knowledge of his time. In 1739, he was appointed head of the Parisian Jardin du Roi (later the Jardin des Plantes). Before that, he was involved in extensive research about sampling and the quality of wood and made his mark in the field of mathematics. In his *Sur le jeu de franc-carreau*, he introduced differential and integral calculus into probability theory and suggested the following Monte Carlo determination of the number π . Imagine a flat plane ruled with parallel lines having unit spacing. Drop at random a needle of length $L \leq 1$ on the plane. What is the probability of observing a crossing?

“At random” needs to be thought about. It seems to mean that the centre of the needle is uniformly placed with respect to the set of lines and that the angle θ that the needle makes with the direction of the lines is uniform in the interval $(0, 2\pi)$ or by symmetry in the interval $(0, \pi)$. There will be a crossing if and only if the distance x from the centre of the needle to the nearest line is less than $L/2 \sin \theta$. From that, Buffon derived the probability $2L/\pi$ for a crossing. So, making some trials of tossing a needle yields an estimate of the definite number π . Without careful modification, however, this first Monte Carlo simulation is not an efficient way of computing numerical constants like π .

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20.3 Dissipative Particle Dynamics Simulations of Vesicle Fusion

Dissipative particle dynamics (DPD) is a coarse-grained simulation algorithm similar to molecular dynamics but able to operate at orders of magnitude larger length and time scales. DPD was invented in 1992 [17] with the aim of simulating a fluid that obeyed the Navier–Stokes equations for hydrodynamic flow without having to calculate all atom–atom interactions as is required in MD. It was later refined by others [8, 13], and the scheme described by Groot and Warren [13] is still the most commonly used one.

The DPD method relies on reducing the number of degrees of freedom that must be integrated in the equations of motion and using soft forces for which a longer integration step size can be used. Most biological systems are embedded in water, and most of the time in a typical MD simulation of a biological system is spent

calculating the forces between atoms, almost all of which belong to water molecules. In a DPD simulation of pure water, several water molecules are grouped together into one water *particle*, and the forces between these particles are constructed so as to reproduce the thermodynamic state of water at room temperature. In this way, a three- to tenfold reduction in the number of particles that must be followed is obtained. Second, the forces between the particles are soft, unlike the Lennard-Jones forces between atoms in MD simulations, and this allows a larger integration step size to be used, which gains a further speedup of several orders of magnitude. The cost of this speedup is a loss of accuracy for small length and time scale motions, such as bond vibrations or hydrogen bond formation, and the need to parametrize the (many) interaction parameters in the DPD forces manually. However, at length and time scales much larger than the atomic, such as are involved in vesicle shape fluctuations or fusion, the neglected small-scale details are expected to be irrelevant. Instead of describing the algorithm in further detail here, we present a brief overview of its use in one instance and refer the interested reader to two review articles [36, 40] and the original literature cited therein.

It was early recognized that DPD could be used to simulate lipid bilayers [34, 39] with almost the same accuracy for the membrane's material properties as computationally more demanding MD simulations [10]. The equilibrium material properties of the simulated membranes (surface tension, elastic stretch modulus, bending modulus and structural properties) are found to be very similar using the two methods. The extension of DPD to simulating membranes containing mixtures of different types of lipid and the dependence of the membrane's material properties on the lipid molecular structure soon followed [18]. Subsequent development of DPD models of lipid bilayers allowed them to be used to study non-equilibrium processes, such as exocytosis or vesicle fusion, and domain formation in mixed membranes [21].

Exocytosis is a fundamental process in living cells that allows cells to transport material across their PM into the extracellular space. Essentially small vesicles fuse with the PM and open a pore through which their contents escape to the extracellular space. It is used both for disposing of waste material and for the release of molecules such as hormones. It requires several highly regulated and precisely timed stages. First, a vesicle is filled with the material to be released and transported to the PM where it is held until the signal for fusion is received. On receipt of the signal, the vesicle fuses with the membrane and undergoes a complex molecular rearrangement, which is still not entirely understood, as a result of which a small pore is created in the closely apposed lipid bilayers of the vesicle and PM. This pore then increases in size to release the vesicle's contents to the outside of the membrane. The pore may increase without limit or only up to a certain size and then shrink again. Once the contents (or a portion thereof) have been released, the vesicle is recycled for further use.

A key stage of exocytosis that is not yet well understood is the final formation of the fusion pore between the vesicle membrane and the PM. This process also occurs in the fusion of transport vesicles to the membrane of internal organelles and is therefore of general interest beyond the specific example of exocytosis. The essential conundrum in understanding how vesicle fusion occurs lies in the nature of

the lipid bilayer membrane that surrounds both the vesicle and the target membrane with which it is to fuse. These membranes are constructed to be resistant to breaking, and the hydrophobic effect ensures that any small pore that forms in the membrane is swiftly eliminated as the lipid molecules diffuse rapidly in and fill the gap. Given that the membranes are so reluctant to allow pores to form, how is the fusion pore produced? Proteins play an important role in organizing the membranes, bringing them together and, finally, in driving them to fuse. Recently, computer simulations have been used to explore what the proteins may be doing in creating the fusion pore in two closely apposed membranes. Until experimental resolution reaches down to the hundreds of nanoseconds and sub-micron scales, we cannot directly observe the fusion pore form. But the simulations may help us to fill in the gaps in our experimental view. The simulated model systems embody the sum of our knowledge about the system and act as a kind of mathematical microscope, revealing details and connections that we may have overlooked (see [Chapter 6](#) for more details). By comparing the models with experimental results, we can refine our knowledge and obtain a more accurate picture of how fusion takes place at the molecular level.

The final stage of vesicle fusion starts when the vesicle is positioned a few nanometres from the target membrane. Proteins called SNAREs act both as anchors in the membranes and as regulators to control the precise conditions under which the fusion pore forms. Different types of SNARE protein are present in the vesicle membrane and the target membrane. Experiments have shown that SNARE proteins are anchored in the membranes by short peptide sequences that appear to act as (almost) rigid rods. They also have extracellular parts that recognize and bind to each other, pulling the vesicle towards its target membrane.

One of the simplest means of producing a pore in a membrane is to stretch it. Consequently, the simplest model of fusion pore formation is to assume that the membranes are stretched by some means and subsequently relax the tension by fusing and opening up a pore. Several conditions are required for this to be successful. The membranes must be closely apposed (as they are in the cell) before the tension is applied as they will otherwise just burst independently and not fuse. The required tension must not be so large that it is infeasible for proteins to exert it on the sub-microsecond physical time scale. Finally, the pore formation should be reliable in the sense that it is robust against the perturbations of the random thermal motion of the lipids and proteins. A recent series of simulation studies [9, 12, 35, 36, 41] have shown that these conditions can be satisfied and that applying forces that are spatially and temporally localized to two closely apposed model membranes yields reliable fusion.

Early attempts to drive the fusion of a vesicle to a planar lipid bilayer patch [35] raised the tension globally in both the vesicle and the planar membrane. The fusion protocol consisted of placing a tense vesicle near a tense planar membrane, with the two tensions being used as control parameters. As the simulation progressed, the random motion of the vesicle lipids caused them to touch and then merge with the planar membrane. Once the membranes had merged slightly, the vesicle lipids rapidly moved into the more tense planar membrane, and the subsequent disordering

effect led to the fusion pore formation. Fusion was observed for a range of membrane and vesicle tensions. However, the results showed that other outcomes are also possible, and their probability is tension dependent. If the tension is too low, the membranes just adhere to each other, whereas if the tension is too high, one or other of the membranes may rupture before a fusion pore appears. Even for tensions that produce some fusion events, alternative outcomes also occur with quite a high frequency. This study showed that a global tension in the membranes is not a reliable means of driving fusion pore formation. A subsequent study of the fusion of two vesicles using an alternative parameterization of the membrane's material properties showed that the pathway of fusion is tension dependent [9]. At low tensions, the vesicles first adhere and then the adhesion patch subsequently ruptures to produce the fusion pore, while at high tensions the membranes form a stalk that then opens a transmembrane pore that rapidly expands radially. The fusion process was found to have two energy barriers that had to be crossed, each of which was of the order of 12 kT.

The usefulness of particle-based simulations of fusion in revealing the importance of molecular rearrangements was demonstrated by another study that followed the fusion of a vesicle to a planar membrane patch [12]. Using the same fusion protocol as above, in which both the vesicle and the planar membrane are under tension and placed close together, the fusion event started with the vesicle first adhering to the planar membrane and then a few lipids from the vesicle adopted a “splayed” conformation in which one of their tails is embedded in each membrane. This led to more lipids crossing from the vesicle and forming a disordered patch within which a small (a few nanometres) patch of hemi-fused bilayer appears that eventually ruptures to form the fusion pore. Three energy barriers were extracted from the fusion events that were all of the order of 8–15 kT. A key result of this work was that the splayed lipid conformation, which had earlier been postulated as relevant to fusion [16], was observed in the simulations but had not been addressed in previous theoretical models.

Protein-mediated vesicle fusion is unlikely to use a global tension, and some simulations have been made of localized tension-induced fusion. Because SNARE proteins are anchored in the membranes, and these anchors have been shown to act as force transducers [23], it was suggested [36] that the SNAREs apply forces and torques to the apposed membranes leading them to fuse. Figure 20.1 shows a snapshot taken from one vesicle fusion event simulated with DPD. The vesicle has a diameter of 28 nm, and the planar membrane patch has a linear dimension of 100 nm. Both the vesicle and the membrane patch are initially tensionless, and six cylindrical, transmembrane barrels are embedded in a hexagonal arrangement in each membrane. The barrels are placed around the point of closest approach of the vesicle and planar membrane. A sequence of external forces is then applied to the barrels so as to bend and stretch the membranes inducing the vesicle to fuse. Such a protocol was found to lead to reliable fusion, in the sense that alternative outcomes such as membrane rupture or hemifusion were not observed, but the work required to induce pore formation was quite large, approximately 90 kT per protein.

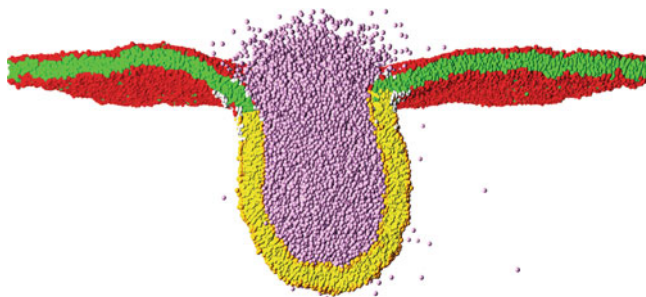


Fig. 20.1 Cross-sectional view of a 28-nm diameter vesicle fusing with a $100 \times 100 \text{ nm}^2$ planar lipid bilayer immersed in solvent. The solvent particles, which represent water, are invisible outside the vesicle, but those initially inside the vesicle are made visible so that their expulsion through the fusion pore can be seen. The vesicle contains 5,900 lipids and the planar membrane 28,000 lipids, and the simulation box contains over 3 million particles in total. Each lipid consists of three hydrophilic head beads to which two linear chains of four hydrophobic beads each are connected. Both the vesicle and the planar membrane are initially tensionless, and the pore is created by applying external forces to embedded transmembrane “barrels” in the two apposed membranes (not visible). The snapshot is taken about 300 ns after initial contact of the vesicle to the membrane, and only a few internal water particles have emerged through the vesicle’s membrane before the fusion pore forms. Simulating one complete fusion event requires about 4 cpu days on a single processor. Further details of this fusion protocol can be found in Shillcock and Lipowsky [36].

More details of these fusion simulations can be found in Shillcock and Lipowsky [36].

Further optimization of the membrane and protein material properties may lower the work required for fusion, and encouragingly the number of proteins needed (6) and their typical separation in the membrane were in agreement with *in vitro* experiments [42]. The hypothesis that SNAREs exert an explicit force on their attached membranes, and that this force helps drive the fusion pore formation, has lately received experimental support [1]. Another simulation study [41] has gone further in modelling protein-induced fusion by including the extracellular piece of the SNARE proteins as well as their transmembrane anchors. The fusion pore was created by the forced binding of the extracellular pieces of the model proteins that pulls the membranes together and stretches a small patch at the centre of the ring of proteins until it forms a pore.

This example of vesicle fusion provides a case study of the ability of coarse-grained simulations to iteratively include more and more details of a dynamical system until predictions can be made. In the specific case of fusion, the number and height of the energy barriers, the probable number of protein complexes required to create a fusion pore and the work done by the proteins can be compared to experiments, and the models further refined. Crucially, the simulations reveal the molecular rearrangements that occur along the path to fusion and do not rely on a priori intuition as to the important steps in the process. In this way, they provide information that aids in visualizing the complex molecular dance that occurs inside a cell [33].

20.4 Conclusions and Outlook

On a time scale of milliseconds to seconds, a cell creates new proteins, brings material into the cytoplasm via endocytosis and expels material via exocytosis. If we take one vesicle fusion event as the unit of time, say $1\ \mu\text{s}$, and we imagine following a cell's behaviour for 1 s, we will observe approximately 1,000,000 fusion events. These define a mean fusion time and variance and lead to a net flux of material into or out of the cell that results in changes of the cellular plasma membrane area and its volume. These have to be kept constant on average, so the cell has to continually regulate endo- and exocytotic events. This requires monitoring and regulating processes that span length and time scales far beyond what can be represented in a single type of simulation or model.

The difficulty of trying to represent cellular dynamics in a simulation can be illustrated as follows. A highly simplified schematic of a cell is shown in Fig. 20.2. It

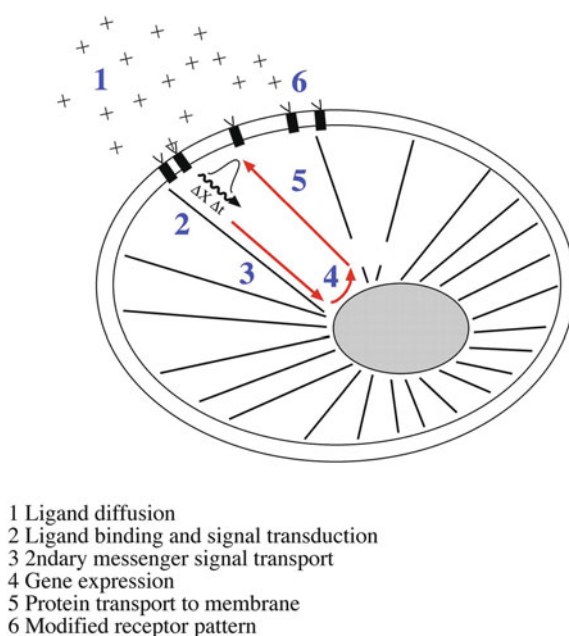


Fig. 20.2 Illustration of the transport occurring on different length and time scales that is relevant for computer simulation of cell signalling processes. First, freely diffusing ligands approach the cell and bind to receptors on the plasma membrane (1). This causes a signal to be transduced across the PM (2) which is then propagated through the cytoplasm to the cell nucleus (3). Gene expression may then be modified resulting in a change in protein concentrations in the cell (4). Newly created proteins are transported back to the PM (5) where they result in a changed response to newly arriving ligands (6). Proteins may also be sent to other locations in the cytoplasm. Of these six processes, it is currently only possible to simulate the first (and possibly the sixth) using particle-based simulations due to the computational demand of simulating lengths from nanometre to microns. Combining different modelling techniques so as to represent, and link up, all the processes represented here is the primary goal of multiscale modelling.

can be divided into three spatio-temporal regions (plasma membrane, cytoplasm, nucleus) between which six transport mechanisms take place. First, signalling molecules diffuse onto the cell's surface, bind to receptors (1) and lead to the release of secondary messengers into the cytoplasm (2). The signal is then carried to the cell nucleus via complex signalling networks (3), where it results in modified gene expression (4). This results in new proteins being created and transported back to the PM (5) or released into the cytoplasm. The new proteins subsequently modify the cell's behaviour, often by changing its sensitivity to further signalling molecules (6). This completes the loop as the cell is now in a new state with a distinct response to the original signal. The length and time scales (ΔX , ΔT) associated with each of these processes are quite distinct. Signal transduction across the PM takes place over a nanometre distance and nanosecond/microsecond time, whereas gene expression can take minutes depending on the size of the proteins. Protein transport from the endoplasmic reticulum, where proteins are produced, to the PM requires them to be moved over micron distances. These processes have to be robust even though the cytoplasmic contents are in continual thermal motion.

In the cytoplasm, proteins and other aggregates move continually under the influence of thermal fluctuations. But they move in a crowded environment, and it is not clear that the *in vivo* conditions correspond to those created *in vitro*. Hence, models of membrane-associated processes must include at least three features: the membrane's material properties and structure, and its dynamic remodelling during vesicle fusion, etc., and the diffusive environment of the near-membrane cytoplasm. The plasma membrane is a composite of a fluid–lipid bilayer and the membrane-associated cytoskeleton. The latter is a triangulated network of filaments with a mesh length of the order of 70 nm, so it is too large for atomistic molecular dynamics simulations. The shape fluctuations of the membrane and the influence of the cytoskeleton are crucial to the stability of the PM. Remodelling of the cytoskeleton also occurs during bacterial entry into a cell or nanoparticle injection. Such processes take place simultaneously with the movement of proteins and transmission of signals through the cell and are deeply connected. Because of the complexity of the interactions, most models of protein–protein interactions involved in cellular signalling have used a simplified scheme adopted from the study of chemical reactions known as reaction rate models.

Chemical reactions are modelled using differential equations in which each reactant and product is represented by its concentration in the reaction volume. It is assumed that all chemical species freely diffuse throughout the whole volume much faster than the rates at which they react. Second, it is assumed that all species are present in large numbers so that their representation by continuously varying concentrations is appropriate. Both these assumptions can fail for specific cellular processes. When they do fail, the properties of the system are strongly influenced by the spatial distribution of the species and their diffusion rates.

The ratio of the time scales for protein diffusion in the cytoplasm of the cell and their reaction rates is important for signal transduction [20]. But understanding the effects of the crowded cytoplasm on the protein diffusion rates is a problem that cannot be solved using continuum theories, and experiments are hard because it is

difficult to track the movement of several species simultaneously. There is even a controversy for the simpler case of protein lateral motion in reconstituted giant unilamellar vesicles [28]. Different experiments predict a $1/R$ dependence on the protein radius R or $\log(1/R)$. The discrepancy has been tentatively attributed by Ramadurai et al. [28] to the use of supported membranes in some of the experiments, showing that sample preparation is not trivial for these systems. Computer simulations of the cellular cytoplasm containing a known mixtures of particles would allow a comparison of the diffusion of particles of known size under various conditions [32]. Comparing these results to experiments using QDs attached to proteins or nanoparticles allows us to estimate the distribution of cytoplasmic constituents. Some protein species may be present only as a few molecules, and so their concentration fluctuates wildly in small regions, and the assumption of smooth concentration changes in reaction rate models fails again [38]. Particle-based simulations can yield quantitative information on the distribution of chemical species (however few) in crowded, spatially irregular environments, such as in the cytoplasmic region near the PM, that may be useful for calibrating some of the parameters in reaction rate models.

Particle-based simulations on state-of-the-art hardware currently require at least a few days of computational effort to follow a single fusion event. Even allowing for an increase in processor speeds with time, it will not be possible to simulate even a fraction of the cellular PM with such methods for many years.

But a combination of parallel simulation codes that can run on clusters of hundreds or thousands of networked processing nodes together with multiscale models [26] may provide a route to modelling cellular dynamics. Each more accurate level of modelling passes data upwards to parametrize the next higher levels [19]. Such a hybrid simulation tool will contain much of the knowledge that is currently stored in quite distinct modelling methodologies and make it easier for functional modules to be inserted or removed at will depending on the desired behaviour. It would form a repository of knowledge of cellular dynamical behaviour that could be used to test hypotheses about processes ranging from bacterial entry into a cell, nanoparticle toxicity, to therapeutic targeting of drug delivery vehicles.

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Chapter 21

What Drives Calcium Oscillations in β -Cells?

New Tasks for Cyclic Analysis

Leonid E. Fridlyand and Louis H. Philipson

Abstract Insulin secretion is initiated by metabolism-dependent depolarization of the plasma membrane and regulated in part by oscillations of the plasma membrane potential, which drive oscillations of cytosolic calcium. Mathematical models have been useful to help understand the key factors underlying these phenomena. Modelling approaches show that candidates for a pacemaker underlying the membrane potential bursts and cytosolic Ca^{2+} oscillations include cyclic changes in the ratio of ATP to ADP, Ca^{2+} concentration in the endoplasmic reticulum or cytosolic Na^+ concentration. However, additional experiments to evaluate the dynamics of integrated signalling systems with cyclic analysis using mathematical models are necessary to better understand this emerging aspect of β -cell physiology.

Keywords β -cell · Electrophysiology · Calcium · Computing modeling · Diabetes · Oscillations

Abbreviations

AP	action potentials
$[\text{Ca}^{2+}]_c$	free calcium concentration
$[\text{Ca}^{2+}]_{\text{ER}}$	free calcium concentration in ER
CRAC	Ca^{2+} release-activated current
ER	endoplasmic reticulum
IP_3R	inositol 1,4,5 triphosphate receptor channels
K_{ATP}	ATP-sensitive K^+ channels
K_{Ca}	Ca^{2+} -activated K^+ channels
PM	plasma membrane
VDCC	voltage-dependent Ca^{2+} channel

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21.1 Introduction

Insulin secretion from the pancreatic β -cell induced by physiological secretagogues, primarily glucose, is mediated by an elevated cytosolic free calcium concentration ($[\text{Ca}^{2+}]_c$). The metabolism of glucose raises the ratio of ATP to ADP, closing the ATP-sensitive K^+ (K_{ATP}) channels. Closure of these channels leads to plasma membrane (PM) depolarization up to a threshold potential where voltage-dependent Ca^{2+} channels (VDCCs) located on the PM are activated. Ca^{2+} influx through VDCCs leads to an increased $[\text{Ca}^{2+}]_c$, which triggers the exocytosis of insulin-containing granules. There is an extensive literature describing β -cell electrical activity and its relationship to intracellular Ca^{2+} concentration in intact islets of Langerhans and isolated islet cells (for recent review see [23, 24]). Mechanisms of glucose-induced PM depolarization, cytosolic and mitochondrial processes were also considered in previous chapters.

The β -cell membrane is hyperpolarized at resting potential at low glucose levels ($\sim 3\text{--}5$ mM) in islets. When glucose metabolism induces an increased potential across the membrane, a resultant electrical activity of the pancreatic β -cell is usually organized into slow depolarizing waves, called bursting, with a plateau from which action potentials (AP) rapidly fire. They are separated by quiescent (resting) periods at potentials below the AP threshold. This bursting phenomenon regenerates as long as the glucose concentration is elevated and results from the metabolic processes and electrical activity of ion channels and pumps localized on the β -cell plasma membrane [3, 24].

Rapid depolarization at the beginning of the burst and a repolarization at the end result in opening and closing of VDCCs. This leads to cytosolic Ca^{2+} oscillations which are synchronized with cyclical spike-burst activity in response to a rise in extracellular glucose [23, 24]. These Ca^{2+} oscillations are intimately connected to multiple key aspects of β -cell physiology and their regulation continues to be an important area of study.

Increased glucose concentration induces several types of cyclical spike-burst activity and Ca^{2+} oscillations in insulin-secreting β -cells with different periods [5, 8, 23]. Oscillations with a period from several seconds to 1 min are usually denoted as “fast”. Ca^{2+} oscillations with a period ranging from 1 to several minutes and long bursts are designated as “slow”. Mixed (or compound) Ca^{2+} oscillations are characterized by fast oscillations superimposed on slow oscillations. This is due to periodic episodes of fast bursts, called compound bursting [5, 9].

Fast electrical bursting and cytoplasmic Ca^{2+} oscillations are usually observed in isolated mouse islets. Slow and mixed cytoplasmic Ca^{2+} oscillations are observed both in islets, single cells and clusters of cells at stimulating glucose concentrations [5, 9, 21].

Various experimental and theoretical approaches support the idea that the AP bursts, when spike activity occurs, and the corresponding $[\text{Ca}^{2+}]_c$ oscillations reflect a periodic depolarization of the plasma membrane [3, 5, 18]. A depolarizing component predominates at the beginning of the burst, but the resultant influx of Ca^{2+} during the burst leads to a progressive increase in outward and/or

a decrease in inward cation currents leading to repolarization and burst termination. Slow depolarization in a resting (silent) phase should lead to burst re-initiation [3, 18, 23].

After the large depolarizing effect of closing K_{ATP} channels, the subsequent generation and termination of the bursts may be determined by small cyclical changes of any plasma membrane current [18]. For this reason, initiation and termination of the bursts result from activation and deactivation of several electrogenic channels, pumps or exchangers which can be modulated in turn by numerous intracellular components. A variety of different proposals and corresponding mathematical models have been advanced underlining the nature of these components.

In this chapter we discuss an analysis of the proposals to explain these phenomena, together with the underlying experimental data and the corresponding mathematical models. Bertram and Sherman [10] have written a historical review on the development of mathematical modelling of β -cell oscillatory processes. For this reason we concentrated on recent developments in this field and new experimental evidence with the goal of understanding how bursting and $[Ca^{2+}]_c$ oscillations can arise. Several illustrative examples are presented below. We consider here only models for individual β -cells that represent behaviour of cells in electrically synchronized normal islets. Models that treat the effect of electrical coupling can also be found (see, for example, [7]).

Bursting and $[Ca^{2+}]_c$ oscillations are easily obtainable phenomena in isolated mouse islets. Oscillatory behaviour can be changed by numerous hormones, small molecule agonists as well as toxins. The study of the changes in bursting or

Fourier Analysis

The idea that you can represent a pure tone by sinusoidal oscillation of a given frequency is at the heart of Fourier analysis. Instead of describing the function in the time domain as

$$P(t) = A \cdot \sin(2\pi ft),$$

where P is a measure of the air pressure, the amplitude A is a measure of the loudness of the tone, and f is the pitch of the tone (the concert pitch is 440 Hz), the tone is represented by a single frequency and amplitude in the frequency domain. More complex functions in the time domain can be built by adding more sinusoidal components.

The relation above is an example of a discrete Fourier transform (DFT). The DFT is especially useful when analysing oscillatory signals with periodicity. Since the discovery of the fast Fourier transform (FFT), this has become the preferred way of time signal analysis.

Further Reading:

Kammler DW (2007) A first course in Fourier analysis. Cambridge University Press, Cambridge

Added by the editors

$[Ca^{2+}]_c$ oscillations could yield a flexible systematic explanation for the action of different signalling molecules on pancreatic β -cells. However, this important approach requires a comprehensive knowledge of the pacemaker mechanisms of these oscillations.

Interestingly, the rhythmic variations in insulin secretion from islets are synchronized with oscillations of the cytoplasmic Ca^{2+} concentration [23, 34]. Whereas pulsatility appears to be a natural function of islets both in vivo and in vitro, it has been hypothesized that the disruptions in rhythmic function may be an early biomarker of islet dysfunction leading to diabetes [34]. This emphasizes also the need for careful study of these oscillatory processes.

21.2 Schematic Model

The main components that we consider in this chapter as important for generation of bursting and cytoplasmic Ca^{2+} oscillations are summarized in Fig. 21.1. The “complex model” of processes (from [19]) was used for the following simulations. This model is available for direct simulation on the website “Virtual Cell” (www.nrcam.uchc.edu) in “MathModel Database” on the “math workspace” in the library “Fridlyand” with name “Chicago 1”.

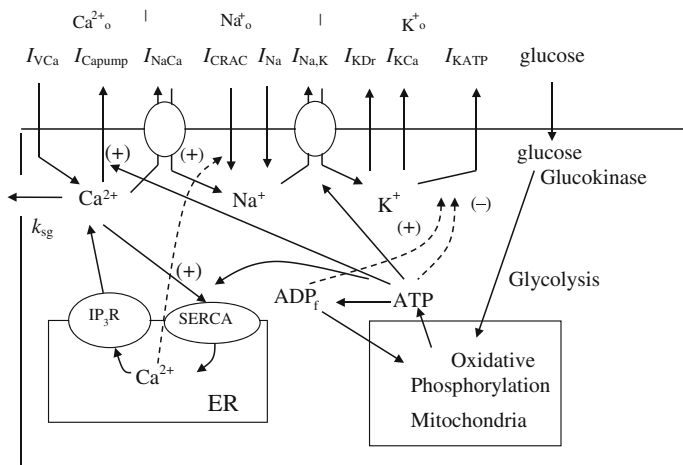


Fig. 21.1 General scheme of the main processes involved in bursting and intracellular Ca^{2+} oscillations in pancreatic β -cells. *Top*: plasma membrane currents: voltage-dependent Ca^{2+} current (I_{VCa}), a calcium pump current (I_{Capump}), Na^+/Ca^{2+} exchange current (I_{NaCa}), Ca^{2+} release-activated current (I_{CRAC}), inward Na^+ currents (I_{Na}), a sodium–potassium pump current (I_{NaK}), a delayed rectifying K^+ current (I_{KDr}), the small conductance Ca^{2+} -activated K^+ current (I_{KCa}), ATP-sensitive K^+ current (I_{KATP}). k_{sg} is a coefficient of the sequestration rate of Ca^{2+} by the secretory granules, SERCA is a calcium pump in the ER, Ca^{2+} leaks from ER through the IP_3 receptor (IP_3R). “ATP” is the free cytosolic form of ATP, ADP_f is the free cytosolic form of ADP. Signals originating from fuel metabolism increase cytosolic calcium.

The differential equation describing time-dependent changes in the plasma membrane potential (V_p) is the current balance equation:

$$C_m = \frac{dV_p}{dt} = I_{VCa} + I_{Capump} + I_{NaCa} + I_{CRAC} + I_{Na} + I_{NaK} + I_{KDr} + I_{KCa} + I_{KATP} \quad (21.1)$$

where C_m is the whole cell membrane capacitance. The plasma membrane currents are listed in Fig. 21.1.

Equations for $[Ca^{2+}]_c$ dynamics can also be written as in our model [19]:

$$\frac{d[Ca^{2+}]_c}{dt} = f_i \left[\frac{-I_{VCa} + 2I_{NaCa} - 2I_{Ca\ pump}}{2FV_i} - J_{er,p} + \frac{J_{out}}{V_i} \right] - k_{sg}[Ca^{2+}]_c \quad (21.2)$$

where f_i is the fraction of free Ca^{2+} in cytoplasm, F is Faraday's constant, V_i is the effective volume of the cytosolic compartment, $J_{er,p}$ is flux into the endoplasmic reticulum (ER) through SERCA pumps per cytosol volume, J_{out} is a Ca^{2+} leak current from the ER per whole cell and k_{sg} is a coefficient of the sequestration rate of $[Ca^{2+}]_c$.

The voltage-dependent Ca^{2+} channel conducts Ca^{2+} ions into the cell, which raises the transmembrane voltage, V_p , whereas the K^+ channel gates efflux of K^+ and restores V_p to a low level. The temporal interaction of the two channels is sufficient to explain the repetitive spiking observed in β -cells (see [10, 18]). However, an inclusion of additional components was necessary to explain a burst behaviour.

21.3 $[Ca^{2+}]_c$ as the Pacemaker Component

Initially, a mechanism with a $[Ca^{2+}]_c$ feedback effect was proposed to underlie generation of bursts and $[Ca^{2+}]_c$ oscillations. According to this hypothesis Ca^{2+} influx during the active phase would cause a slow rise in $[Ca^{2+}]_c$, which activated Ca^{2+} -activated K^+ (K_{Ca}) channels (current I_{KCa} in Eq. (21.1)) that in turn repolarized the plasma membrane potential until a critical level was attained. This would shut down the spiking and inactivate VDCCs, leading to a resting phase. A slow decrease in $[Ca^{2+}]_c$ levels in a resting period would lead to decreased current through K_{Ca} channels, re-initiating PM depolarization and of the start of a new burst. In this case a cyclical change in $[Ca^{2+}]_c$ is a candidate for an islet pacemaker for burst behaviour and $[Ca^{2+}]_c$ oscillations per se [4]. For a description of mathematical models incorporating the cyclical activation and deactivation of K_{Ca} channels by bursting-induced elevations in $[Ca^{2+}]_c$, see Bertram and Sherman [10].

One problem with this hypothesis is that $[Ca^{2+}]_c$ should increase slowly during a burst period leading to a slow increase of current through K_{Ca} channels. However, subsequent Ca^{2+} imaging data indicate that the time scale of the $[Ca^{2+}]_c$ change is

short relative to the oscillation period. For this and other reasons, this mechanism for $[Ca^{2+}]_c$ oscillations was ruled out (see [10]).

21.4 Role of [ATP]/[ADP] Ratio as Pacemaker

After the large inward currents of K_{ATP} channels are mostly inhibited following glucose metabolism, their small remaining conductance is still comparable with the conductance of other channels, exchangers and pumps [33]. K_{ATP} channel regulation can thus participate in the subsequent generation and termination of the bursts. Cyclical changes in the K_{ATP} channel conductance were proposed as a mechanism underlying oscillatory behaviour of β -cells [25, 31, 33]. One possibility is that the [ATP] to [ADP] ratio slowly decreases when $[Ca^{2+}]_c$ increases during a burst, leading to a slow opening of the remainder K_{ATP} channels and PM repolarization. The opposite idea is that the [ATP]/[ADP] ratio gradually increases during a silent phase, when $[Ca^{2+}]_c$ decreases, leading to further closure of K_{ATP} channels and plasma membrane depolarization. New bursts then are initiated when the plasma membrane depolarizes up to the threshold level [25, 31]. Indeed, the [ATP]/[ADP] ratio drops when $[Ca^{2+}]_c$ rises and increases when $[Ca^{2+}]_c$ falls [1, 15] indirectly supporting the proposal that $[Ca^{2+}]_c$ oscillations can therefore evoke oscillations in [ATP]/[ADP] ratio and K_{ATP} channel conductance.

Several mathematical models underlining the mechanisms of the [ATP]/[ADP] ratio changes and attendant $[Ca^{2+}]_c$ oscillations were proposed, as follows. Empirical equations for [ATP]/[ADP] ratio changes were introduced by Smolen and Keizer [33]. In this formulation the [ATP]/[ADP] ratio decreases slowly with increased $[Ca^{2+}]_c$ and increases with decreased $[Ca^{2+}]_c$ in simulations using their empirical equation. Periodic changes in the [ATP]/[ADP] ratio and the corresponding K_{ATP} channel conductances, bursting and $[Ca^{2+}]_c$ oscillations were simulated using this model.

Several mechanistic hypotheses underlying the mechanism of how $[Ca^{2+}]_c$ effects the [ATP]/[ADP] ratio were also proposed. A slow decrease in the [ATP]/[ADP] ratio with increased $[Ca^{2+}]_c$ during bursts can result from either stimulation of ATP hydrolysis or inhibition of ATP production.

$[Ca^{2+}]_c$ *activated ATP consumption*. Ca^{2+} activates Ca^{2+} pumps on the plasma membrane and in the endoplasmic reticulum (ER), as well as other intracellular reactions that use ATP. In these cases increased $[Ca^{2+}]_c$ can increase ATP consumption and decrease the [ATP]/[ADP] ratio. An opposite process which increases the [ATP]/[ADP] ratio with decreased ATP consumption can occur with decreased $[Ca^{2+}]_c$ [19, 31]. We illustrate this mechanism using a mathematical model [19] that includes simulation of ATP consumption due to the work of Ca^{2+} pumps in the plasma membrane and ER as well as Ca^{2+} -activated ATP consumption in some cytosolic processes. The simulated phase relations are represented in Fig. 21.2 for the conditions when free ADP concentration in the cytoplasm (and the corresponding [ATP]/[ADP] ratio) is the main slow pacemaker parameter in this

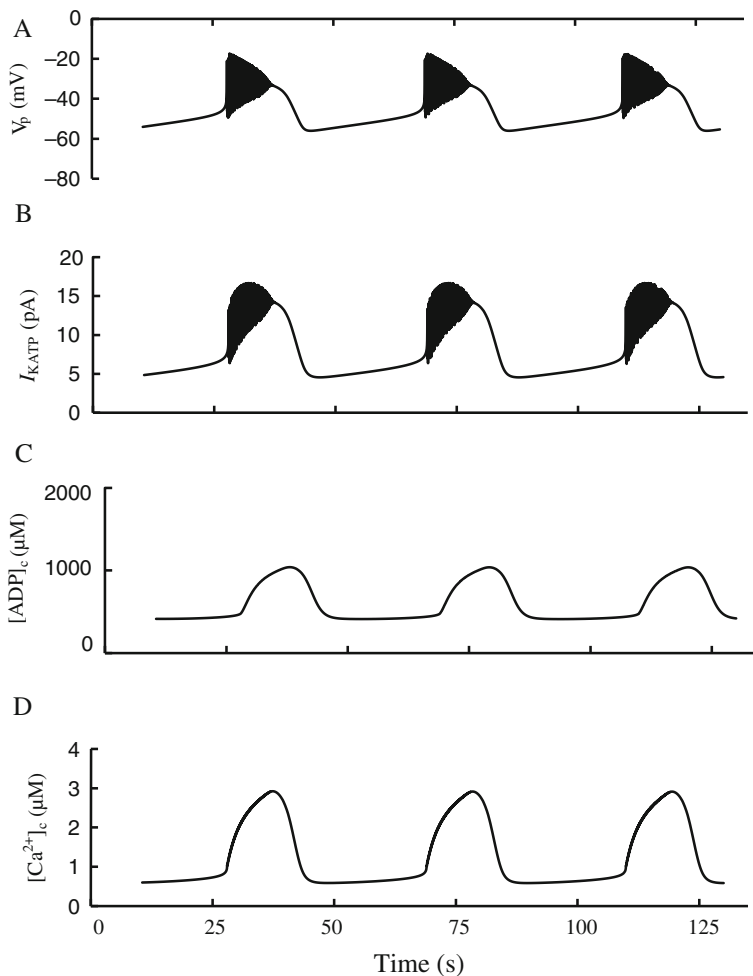


Fig. 21.2 Slow ATP/ADP ratio changes as a pacemaker. Burst behaviour and the oscillation patterns are illustrated. It was simulated using model [19] for conditions when changes in ATP/ADP ratio and corresponding I_{KATP} are the main component determining the membrane potential cyclic variations by setting $g_{mCRAN} = 2 \text{ pS}^{-1} \text{ mV}$, $g_{mKATP} = 70,000 \text{ pS}$, $g_{mKCa} = 150 \text{ pS}$, $g_{mVCa} = 1000 \text{ pS}$, $k_{ADP} = 0.001 \text{ ms}$, $k_{ATP} = 0.00002 \text{ ms}$, $k_{ATP,Ca} = 0.00001 \text{ } \mu\text{M}^{-1} \text{ ms}^{-1}$, $P_{CaER} = 0.05 \text{ } \mu\text{M}^{-1} \text{ ms}$, $P_{IP3} = 0.0004 \text{ pl}^{-1} \text{ ms}$, $P_{NaK} = 80 \text{ fA}$. All other parameter settings are as in Fig. 3 and tables from [19]. (a) Action potential (V_p), (b) I_{KATP} , (c) free $[\text{ADP}]_c$ and (d) $[\text{Ca}^{2+}]_c$.

complex model. In this case a fast rise in $[\text{Ca}^{2+}]_c$ during the active phase leads to increased Ca^{2+} -activated ATP consumption in the cytoplasm. Free ADP increases (and $[\text{ATP}]/[\text{ADP}]$ ratio decreases) slowly, opening K_{ATP} channels. This in turn leads to a slow I_{KATP} increase and plasma membrane repolarization, damping of potential spikes and then a corresponding decrease in $[\text{Ca}^{2+}]_c$. These processes have

opposite directions after decreased $[Ca^{2+}]_c$ in the silent phase. K_{Ca} channels serve to damp depolarization and terminate increased $[Ca^{2+}]_c$ arising from the initial part of the active phase.

$[Ca^{2+}]_c$ suppressed ATP consumption. An earlier proposal suggested that the uptake of Ca^{2+} by β -cell mitochondria suppressed the rate of production of ATP because Ca^{2+} decreased oxidative phosphorylation resulting in an energy-dissipative effect. This proposal also leads to a decreased $[ATP]/[ADP]$ ratio with increased $[Ca^{2+}]_c$ as in the first case. This allows the possibility of simulating $[Ca^{2+}]_c$ oscillations through the cyclical changes in $[ATP]/[ADP]$ ratio [25]. However, recent experimental data favour the opposite idea, as for example it was pointed out that “the primary role of mitochondrial Ca^{2+} is the stimulation of oxidative phosphorylation” [12].

Hypotheses have been proposed to explain the mechanism of $[ATP]/[ADP]$ ratio oscillations through glycolytic oscillations. This was suggested to occur by the positive feedback of the glycolytic enzyme phosphofructokinase product (fructose 1,6-bisphosphate) on phosphofructokinase activity and subsequent depletion of substrate [36]. Mathematical models were constructed based on this mechanism [9, 37].

However, an explanation of the slow $[Ca^{2+}]_c$ oscillations on the basis of changes in K_{ATP} channel conductivity involves difficulties. According to this mechanism the conductance of the K_{ATP} channels should oscillate during bursting electrical activity following changes in the $[ATP]/[ADP]$ ratio. However, K_{ATP} channel blockers such as sulphonylurea, which result in essentially complete I_{KATP} block, do not stop bursting and slow Ca^{2+} oscillations. Sulphonylurea drug can even induce Ca^{2+} oscillations [27, 30]. Existence of slow $[Ca^{2+}]_c$ oscillations was also found in a knock-out mouse lacking functional K_{ATP} channels [17, 29]. These results argue against an important role for dynamic changes in K_{ATP} channel conductivity and, consequently, in $[ATP]/[ADP]$ ratios in the generation of slow Ca^{2+} oscillations.

21.5 ER Ca^{2+} as a Pacemaker Component

The endoplasmic reticulum (ER) is a high-affinity and high-capacity organelle for calcium storage. The β -cell ER sequesters Ca^{2+} when the cytosolic Ca^{2+} level is high and releases it when $[Ca^{2+}]_c$ is low. Ca^{2+} enters the ER via P-type ATPases (SERCA pumps, primarily SERCA2/3 in β -cells) using ATP and exits through two ER Ca^{2+} channels: the inositol 1,4,5 triphosphate receptor channels (IP₃R) and the ryanodine receptor channels (Fig. 21.1) [2, 26, 35].

The ER influences Ca^{2+} dynamics in many cell types. The ER can play an important role in creating Ca^{2+} oscillations through activation of IP₃R on ER membranes [6]. Several mathematical models were made underlining this mechanism for different, usually electrically unexcitable types of cells, resulting in Ca^{2+} oscillations if the inositol 1,4,5 triphosphate concentration is in the correct range [6, 32]. However, β -cells are excitable cells and Ca^{2+} ion influx from the extracellular space through

VDCCs and pumping by PM Ca^{2+} pumps, along with internal sequestration in ER stores, are the principal regulators of cytoplasmic Ca^{2+} homeostasis in these cells [2, 19].

In β -cells, depletion of intracellular Ca^{2+} stores activates a Ca^{2+} release-activated current (CRAC) which represents an inward cation current leading to depolarization that potentiates glucose-induced Ca^{2+} influx through VDCCs [11, 14, 30, 38]. This current represents Na^{+} influx through nonselective plasma membrane cation channels, which have been described in insulin-secreting β -cells [14, 30].

Several mathematical models simulate bursting and corresponding $[\text{Ca}^{2+}]_c$ oscillations on the basis of this effect. Empirical equations for activation of CRAC

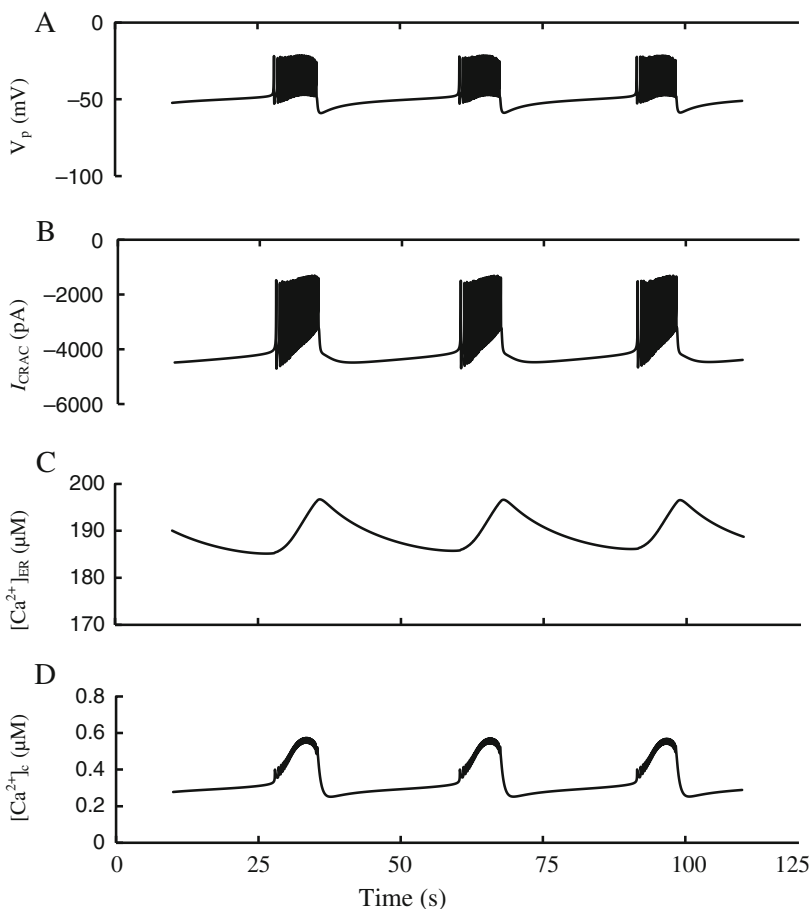


Fig. 21.3 Slow $[\text{Ca}^{2+}]_{\text{ER}}$ changes as a pacemaker. Simulations were made as in Fig. 21.2. Cyclic changes in $[\text{Ca}^{2+}]_{\text{ER}}$ (and corresponding changes in I_{CRAN}) are the main slow parameter in mechanism of Ca^{2+} oscillations by setting $g_{\text{mCRAN}} = 0.85 \text{ pS}^{-1} \text{ mV}$, $g_{\text{mKATP}} = 10,000 \text{ pS}$, $g_{\text{mVCa}} = 600 \text{ pS}$, $k_{\text{ADP}} = 0.0003 \text{ ms}$, $P_{\text{NaK}} = 200 \text{ fA}$. (a) Action potential (V_p), (b) I_{CRAC} , (c) $[\text{Ca}^{2+}]_{\text{ER}}$ and (d) $[\text{Ca}^{2+}]_c$.

currents with decreased $[Ca^{2+}]_{ER}$ were introduced by Bertram et al. [11] and Chay [13]. CRAC increased with a decreased $[Ca^{2+}]_{ER}$ and vice versa using these special empirical equations. Periodic changes of $[Ca^{2+}]_{ER}$, bursting and $[Ca^{2+}]_c$ oscillations were simulated in these models (see also [10]).

We have developed an equation where CRAC represents Na^+ influx through some nonselective cation channels which open with decreased $[Ca^{2+}]_{ER}$ [19]. The proposed mechanism is illustrated in Fig. 21.3. In this simulation a rapid $[Ca^{2+}]_c$ increase at the beginning of the active phase led to increased Ca^{2+} pumping into the ER and slow $[Ca^{2+}]_{ER}$ accumulation with corresponding closure of nonselective cation channels. This decreased inward Na^+ current through these channels resulted in PM repolarization, a termination of spiking and a transition to a silent phase. $[Ca^{2+}]_{ER}$ slowly decreases during a silent phase as a result of exit of Ca^{2+} from ER, leading to increased nonselective cation channel conductance, increased inward Na^+ current and PM depolarization. This resulted in subsequent activation of VDCC and a new burst. In this case $[Ca^{2+}]_{ER}$ is a slow pacemaker component in the mechanism for $[Ca^{2+}]_c$ oscillations.

However, an argument can be made against all $[Ca^{2+}]_{ER}$ -dependent mechanisms for slow $[Ca^{2+}]_c$ oscillations, since it seems to be at odds with data demonstrating that slow oscillations can persist in the presence of thapsigargin, the agent that blocks SERCA and empties the ER stores [2, 19], or in a SERCA 3 knock-out mouse [5].

21.6 Intracellular $[Na^+]_c$ as a Slow Component in a Pacemaker Mechanism

Cytosolic Na^+ concentration ($[Na^+]_c$) changes may play an important role in the generation of slow Ca^{2+} oscillations in β -cells [19, 22]. Several components can regulate Na^+ dynamics in β -cells. The electrogenic Na^+K^+ -ATPase extrudes three Na^+ ions in exchange for two K^+ ions for each molecule of ATP hydrolysed generating a net outward flow of cations through the plasma membrane. This enzyme has high activity in all excitable cells, including pancreatic β -cells, since it maintains the high K^+ concentration in the cytoplasm [28]. Like most other cells, the β -cells are equipped with a Na^+/Ca^{2+} exchanger, an electrogenic transporter located on the PM that couples the exchange of 3 Na^+ for 1 Ca^{2+} [20]. A change of cytoplasmic Na^+ concentration ($[Na^+]_c$) will lead to changes in the inward and outward currents and therefore in the PM potential.

We proposed a mechanism where $[Na^+]_c$ is a dynamic pacemaker variable that can govern bursts and slow $[Ca^{2+}]_c$ oscillations even though K_{ATP} channels or CRAC do not change their activity [19]. Figure 21.4 illustrates the proposed mechanism using our mathematical model that includes a description of Na^+K^+ -ATPase, Na^+/Ca^{2+} exchanger and $[Na^+]_c$ dynamics. The following mechanism was responsible for bursting and Ca^{2+} oscillations: increased $[Ca^{2+}]_c$ during a burst period

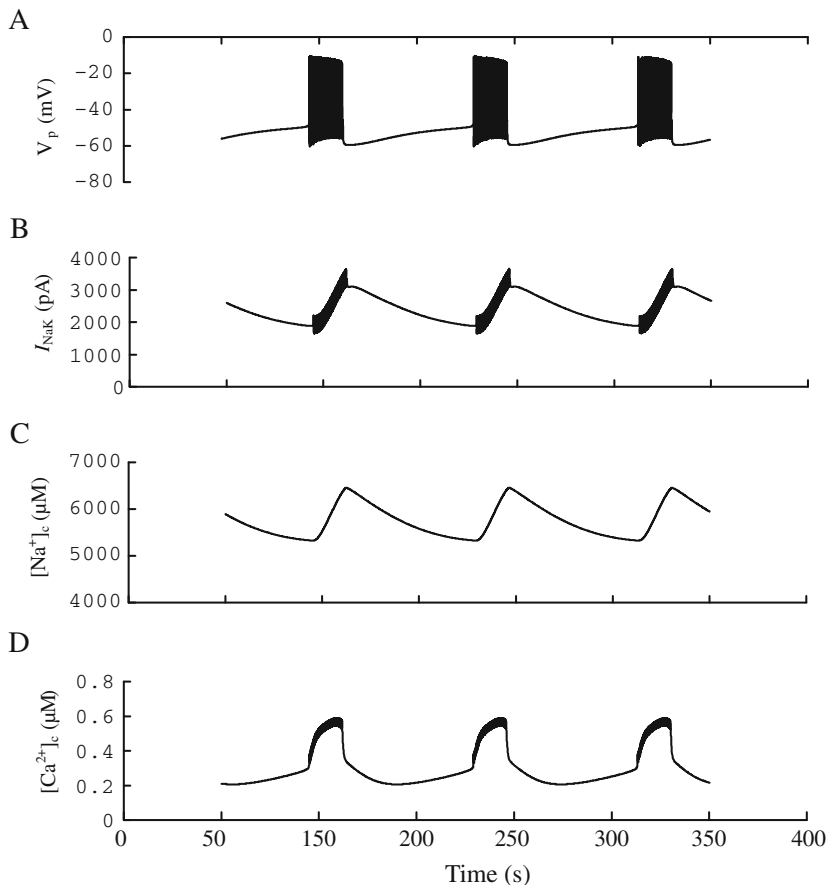


Fig. 21.4 Slow $[Na^+]_c$ changes as a pacemaker. Typical computer simulations were made as in Fig. 21.2. Slow bursting and the oscillation patterns of Ca^{2+} and Na^+ are illustrated. Cyclic changes in $[Na^+]_c$ (and corresponding changes in I_{NaK}) are the main slow parameter in mechanism of Ca^{2+} oscillations by setting $K_{ADP} = 0.0003$ ms, $P_{leuk} = 0.0004$ pl^{-1} ms, $P_{NaK} = 1000$ fA. All other parameter settings are as in Fridlyand et al. [19]. (a) Action potential (V_p), (b) I_{NaK} , (c) free $[Na^+]_c$ and (d) $[Ca^{2+}]_c$.

activates Na^+ influx through Na^+/Ca^{2+} exchangers. The resultant increase in intracellular $[Na^+]_c$ leads to a slow increase of outward current through electrogenic $Na^+ - K^+$ pumps (I_{NaK} in Fig. 21.1) with corresponding plasma membrane repolarization and re-enters a silent phase. Decreased $[Ca^{2+}]_c$ during a silent phase leads to a slow decrease in $[Na^+]_c$ because Na^+ influx through Na^+/Ca^{2+} exchanger decreases. This leads to a decrease of outward current through electrogenic $Na^+ - K^+$ pumps and then in turn to plasma membrane depolarization and an activation of a burst. A correlation of the model simulations with experimental data shows that the suggested mechanism with $[Na^+]_c$ changes can explain bursting and slow $[Ca^{2+}]_c$

oscillations [19]. However, this mechanism of oscillations remains incompletely studied.

21.7 Mechanistic Interactions and Compound Patterns of Bursting and $[Ca^{2+}]_c$ Oscillations

Different $[Ca^{2+}]_c$ oscillations can appear together in the compound (mixed) pattern [9]. The fact that the fast and slow modes of oscillation can occur together, as in the compound pattern, or separately, as in the fast and slow patterns, strongly argues that they stem from distinct mechanisms. These mechanisms can be reciprocally linked and are often co-occurring, but can also proceed largely independently of each other. Mixed oscillations also occur in isolated cells, suggesting that this peculiar pattern does not result from the sum of signals produced in distinct β -cells in islets [21].

Modelling approaches show that the compound behaviour can be simulated and favours a scenario where two or more slow variables interact to produce complex oscillations. For example, Bertram et al. [9] simulated the fast oscillations by small variations in K_{ATP} conductance using an empirical equation connecting the $[ATP]/[ADP]$ ratio with $[Ca^{2+}]_c$ changes, whereas the slow $[Ca^{2+}]_c$ oscillations were simulated as metabolic glycolytic oscillations. These two variables interact to produce compound oscillations, consisting of episodes of bursts separated by long periods of silence, or “accordion” oscillations, which consist of fast bursts with a slowly modulated duty cycle. Similar behaviour could be achieved by defining the slow and fast variables in other ways [8]. For example, according to a recent mathematical model by Diederichs [16], slow $[Ca^{2+}]_c$ oscillations in the compound pattern may be a result of a $[Ca^{2+}]_{ER}$ -dependent mechanism while the K_{ATP} -dependent mechanism may be responsible for fast $[Ca^{2+}]_c$ oscillations superimposed on the top of the slow oscillations.

21.8 Summary

The current mathematical models have great explanatory power. Modelling approaches show that cyclic changes in $[ATP]/[ADP]$ ratio, concentration of Ca^{2+} in endoplasmic reticulum or cytoplasmic Na^+ can be pacemakers for bursts and $[Ca^{2+}]_c$ oscillations. However, some of the experimental data are contradictory and often do not support the existing models. Many of the mechanisms that can control β -cell electrical activity and Ca^{2+} handling have not been characterized. A complete identification of physiological variables that drive bursting or Ca^{2+} oscillations in β -cells and the underlying mechanisms remain elusive. Additional dynamic experiments and mathematical models are necessary to more fully understand this emerging aspect of β -cell physiology.

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Chapter 22

Whole-Body and Cellular Models of Glucose-Stimulated Insulin Secretion

Gianna Maria Toffolo, Morten Gram Pedersen, and Claudio Cobelli

Abstract Models of glucose-stimulated insulin secretion are commonly used to measure β -cell function and to gain insight into the biological mechanisms of insulin release. Depending on the scope, the complexity of the model must be chosen appropriately. We present two models of minimal complexity, able to assess β -cell function in an individual during intravenous and oral glucose perturbations, and a comprehensive model of insulin secretion, describing intracellular events. We show how comparison of cellular and minimal models provides insight into the mechanisms underlying the different aspects of the minimal models and yields biological meaning to their indices.

Keywords β -cell · Insulin secretion · Glucose control · Mathematical models

22.1 Introduction

Mechanistic, physiologically based models have been widely used to describe the control exerted by glucose on insulin secretion. They range from minimal (coarse) models which describe the key components of the system at whole-body level, aiming to measure β -cell function in an individual [9, 16, 24, 26, 41], to maximal (fine-grain) models [6, 14, 22, 32, 33] which include a comprehensive description of the process at cellular level, mainly for simulation purposes.

In general terms, model complexity depends on the question being asked: minimal models are intended to quantify processes which are not directly measurable. The rationale is to link the accessible variables, usually plasma concentrations, to the nonaccessible fluxes/parameters of interest, to be identified on dynamic data measured during a perturbation. The system is described at whole-body level, but

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the model is not a large-scale one: not every known substrate/hormone needs to be included because the macro-level response of the system would be relatively insensitive to many micro-level relationships. In addition, because it is not possible to estimate the values of many system parameters from a limited number of in vivo dynamic data, many of the unit processes must be lumped together. Therefore, desirable features of this class of models include (i) physiologically based; (ii) parameters that can be estimated with reasonable precision from a single dynamic response of the system; (iii) parameters that vary within physiologically plausible ranges; and (iv) ability to describe the dynamics of the system with the smallest number of identifiable parameters.

In contrast to minimal models, maximal (fine-grain) models are comprehensive descriptions attempting to implement the body of knowledge about a process at cellular or even subcellular level. This class of models is not intended to be identified, since without massive experimental investigation on a single individual it is not possible to relate with confidence alterations in the dynamics of blood-borne substances to specific changes in parameters of a comprehensive model. This means that these models are not generally useful for the quantification of specific metabolic relationships – their utility is in their ability to formalize the available knowledge, introduce some new hypothesis and investigate the role of individual components via simulation studies.

To illustrate how these two different modelling approaches provide different insight regarding glucose-stimulated insulin secretion, we present two models of minimal complexity, able to assess β -cell function in an individual during i.v. and oral glucose perturbations and a comprehensive model of insulin secretion, describing intracellular events.

22.2 Modelling Issues in Assessing β -Cell Function

Assessment of β -cell function in humans under physiologic conditions has been a challenge due to the feedback nature of the glucose–insulin system, so that plasma insulin and glucose data reflect not only insulin secretion but also insulin action on hepatic glucose production and glucose utilization by peripheral tissues. Other key insulin processes are also involved, such as hepatic insulin extraction and whole-body insulin kinetics, and all these processes should be assessed under physiologic conditions using a single, simple and physiologic test.

We will briefly discuss these issues, with reference to the minimal modelling strategy.

22.2.1 *Glucose–Insulin Feedback Loop*

While several techniques have been proposed to “open” the feedback loop experimentally, such as the glucose clamp technique, the model-based solution is to maintain the glucose–insulin feedback mechanisms active but open the loop

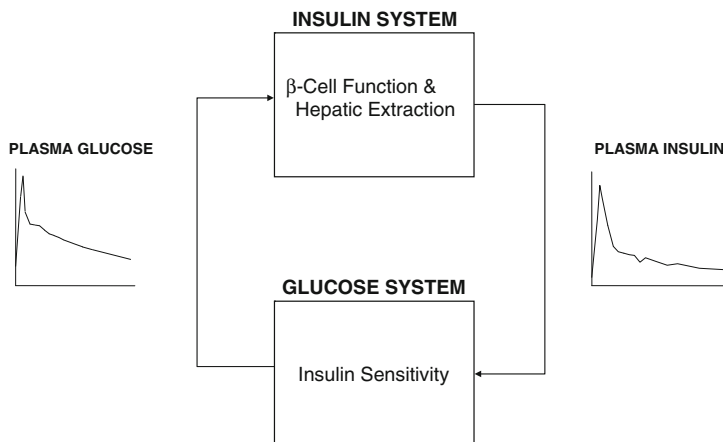


Fig. 22.1 β -cell function is causally related to insulin sensitivity since the glucose system is negatively feedback regulated

mentally by partitioning the whole system into two subsystems (Fig. 22.1) linked by the measurable variables, insulin and glucose concentration. The two subsystems are then modelled separately: for the insulin secretion model, glucose is the (known) input and insulin the output, while for the model of insulin action on glucose production and utilization, insulin is the (known) input and glucose the output. β -cell function is estimated from the insulin secretion model, but then interpreted relative to the prevailing level of insulin action, as discussed in Section 22.4.

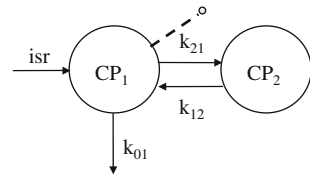
22.2.2 Hepatic Extraction

When inferred from plasma insulin concentrations, insulin secretion cannot be isolated from hepatic insulin extraction since plasma data reflect the fraction of pancreatic secretion which appears in plasma, denoted as post-hepatic insulin secretion and approximately equal to 50% of pancreatic secretion. This problem can be bypassed if C-peptide concentration is measured during the perturbation and used to estimate insulin secretion, since C-peptide is secreted equimolarly with insulin [45], but it is extracted by the liver to a negligible extent [36]. Plasma C-peptide concentration thus reflects C-peptide plasma rate of appearance which, apart from the rapid liver dynamics, is a good measure of C-peptide pancreatic secretion which in turn coincides with insulin pancreatic secretion.

22.2.3 Whole-Body Kinetics

To be identified on plasma C-peptide measurements, the secretion model must be integrated into a model of whole-body C-peptide kinetics. The widely used model

Fig. 22.2 The two-compartment model of C-peptide kinetics



[21] is shown in Fig. 22.2: compartment 1, accessible to measure, represents plasma and rapidly equilibrating tissues and compartment 2 represents tissues in slow exchange with plasma. Model equations are conveniently expressed in terms of C-peptide concentration above basal in the two compartments, denoted as CP_1 and CP_2 (pmol l^{-1}):

$$dCP_1(t)/dt = -[k_{01} + k_{21}] CP_1(t) + k_{12} CP_2(t) + \text{ISR}(t), \quad CP_1(0) = 0, \quad (22.1)$$

$$dCP_2(t)/dt = k_{21} CP_1(t) - k_{12} CP_2(t), \quad CP_2(0) = 0, \quad (22.2)$$

where k_{01} , k_{21} , k_{12} (min^{-1}) are transfer rate parameters and ISR ($\text{pmol l}^{-1} \text{min}^{-1}$) is secretion above basal, normalized by the volume of distribution of compartment 1, to be described according to the models presented in the following section.

Parameters of C-peptide kinetics are usually determined, without loss of accuracy, with the population approach [42].

22.2.4 Intravenous and Oral Glucose Tests

Either intravenous glucose tolerance test, IVGTT, or ingestion of glucose, e.g. an oral glucose tolerance test, OGTT, or a mixed meal is used to perturb the system. The oral perturbations are no doubt more physiological than the intravenous ones with the incretin effect in operation and with the meal being superior to OGTT due to the presence of nutrients, i.e. proteins and fat. Glucose and insulin profiles during the various tests are markedly different: with IVGTT, glucose increases rapidly (within 2–5 min) to the maximum level and then declines to basal, thus rendering evident the biphasic nature of insulin secretion while with oral tests glucose increases in the first 60–90 min and then decreases, with a smoother profile, and the two phases are not clearly separable. Therefore, different models of insulin secretion were developed for the two types of perturbation. They include the same basic ingredients but adapt them to address the different aspects of secretion mechanisms assessed during the two experimental conditions.

22.3 Minimal Models of Insulin Secretion

22.3.1 Intravenous Glucose Tolerance Test

The IVGTT model [41], shown in Fig. 22.3, assumes that insulin secretion rate ISR , appearing as input in the C-peptide kinetic model (22.1) and (22.2), is proportional to the amount of insulin in the secretory granules, X (pmol l^{-1}), which results from the balance between ISR and provision/docking of new insulin secretory granules, Y (pmol min^{-1})

$$ISR(t) = m X(t), \quad (22.3)$$

$$dX(t)/dt = -m X(t) + Y(t), \quad X(0) = X_0. \quad (22.4)$$

Due to the rapid turnover of compartment X ($1/m \sim 2$ min), initial condition X_0 is responsible for first-phase secretion likely representing exocytosis of previously primed insulin secretory granules (commonly called readily releasable). This rapid component of insulin secretion is due to a derivative glucose control, since it is elicited by the rate of increase of glucose from basal up to the maximum occurring after the glucose bolus. The slower second phase derives from Y , that occurs in response to a given (i.e. proportional to) glucose concentration, according to the following equation:

$$dY(t)/dt = -\alpha \{Y(t) - \beta[G(t) - h]\}, \quad Y(0) = 0, \quad (22.5)$$

i.e. in response to an elevated glucose level, Y and thus ISR tends with a delay ($1/\alpha \sim 10$ min in normal subjects) towards a steady-state value linearly related

β -Cell Responsivity Minimal Models

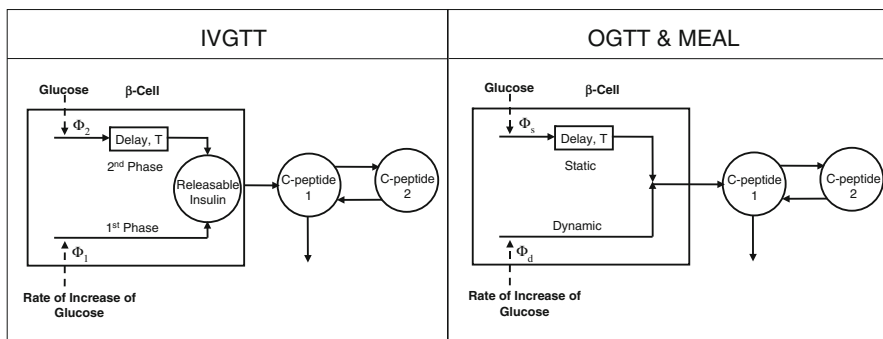


Fig. 22.3 The C-peptide minimal models which allow to estimate β -cell responsivity from an IVGTT (left) and an OGTT and meal (right)

via parameter β (min^{-1}) to glucose concentration G (mmol l^{-1}) above a threshold value h . $1/\alpha$ presumably represents the time required for new “readily releasable” granules to dock, be primed and then be exocytosed.

The meaning of model parameters X_0 , m , β and α is readily envisioned by making reference to a thought experiment of an above-basal step increase of glucose: the rise of glucose induces a first-phase secretion, modelled as a monoexponential decay with time constant $1/m$ from a maximum value X_0 . The total amount of insulin secreted during this phase, obtained by integrating first-phase secretion over time, coincides with X_0 . This is followed by a second-phase secretion due to provision, which tends with time constant $1/\alpha$ towards a steady state which is linearly related to the glucose step size through parameter β .

Given the above mechanistic interpretation of model parameters, it is immediate to define first-phase responsivity to glucose, Φ_1 (dimensionless), as X_0 normalized to the maximum glucose increment ΔG (mmol l^{-1}) and second-phase responsivity to glucose, Φ_2 (min^{-1}), as parameter β :

$$\Phi_1 = X_0/\Delta G, \quad (22.6)$$

$$\Phi_2 = \beta. \quad (22.7)$$

To complete the picture, a basal responsivity index, Φ_b (min^{-1}), can be defined as secretion normalized to glucose concentration

$$\Phi_b = \text{ISR}_b/G_b = k_{01}C_{1b}/G_b, \quad (22.8)$$

where G_b is the end-test glucose concentration.

22.3.2 Oral Glucose Tests

All of the previous model ingredients employed in the IVGTT model were necessary to describe the data, with the exception of the fast turning over insulin releasable pool which is not evident under these conditions [9, 10]. However, at variance with IVGTT where the first phase contributes only during the first 2–5 min, in the oral tests glucose concentration gradually increases during the first 60–90 min, thus requiring a secretion component proportional to the rate of glucose increase. The oral model, shown in Fig. 22.3, thus features a dynamic component, ISR_d , that senses the rate of increase of glucose concentration and a static component, ISR_s , that represents the release of insulin that, after a delay, occurred in proportion to prevailing glucose concentration:

$$\text{ISR}(t) = \text{ISR}_d(t) + \text{ISR}_s(t). \quad (22.9)$$

ISR_d represents the secretion of insulin stored in the β -cells in a promptly releasable form and is proportional to the rate of increase of glucose:

$$SR_d(t) = \begin{cases} k(G) \cdot \dot{G}(t) & \dot{G}(t) > 0 \\ 0 & \dot{G}(t) \leq 0 \end{cases}, \quad (22.10)$$

where

$$k(G) = \begin{cases} k_d \cdot \left(1 - \frac{G(t) - G_b}{G_t - G_b}\right) & G_b \leq G(t) < G_t \\ 0 & \text{otherwise} \end{cases}. \quad (22.11)$$

According to Eq. (22.11), the dynamic control is maximum when glucose increases just above its basal value; it decreases linearly with glucose concentration and vanishes when glucose concentration exceeds the threshold level G_t able to promote the secretion of all stored insulin. For elevated G_t , $k(G)$ approximates the constant k_d .

ISR_s is assumed to be equal to the provision of new insulin to the β -cells, Y ($\text{pmol l}^{-1} \text{ min}^{-1}$):

$$ISR_s(t) = Y(t), \quad (22.12)$$

which is controlled by glucose according to the same equation as for the IVGTT model (Eq. 22.5).

The dynamic responsivity Φ_d (10^9), which is the counterpart of IVGTT first-phase responsivity, is equal to the total amount of insulin released in response to the glucose rate of increase normalized to the maximal increase $G_{\max} - G_b$:

$$\Phi_d = \frac{\int_{G_b}^{G_{\max}} k(G) dG}{G_{\max} - G_b} = \begin{cases} k_d \left[1 - \frac{G_{\max} - G_b}{2 \cdot (G_t - G_b)}\right] & \text{if } G_t < G_{\max} \\ \frac{k_d(G_t - G_b)}{2 \cdot (G_{\max} - G_b)} & \text{if } G_t \geq G_{\max} \end{cases}. \quad (22.13)$$

For elevated G_t , $\Phi_d \approx k_d$. The static responsivity Φ_s (10^9 min^{-1}), which is the counterpart of IVGTT second-phase responsivity, still equals parameter β :

$$\Phi_s = \beta. \quad (22.14)$$

In addition to oral tests, the model described by Eqs. (22.9), (22.10), (22.11) and (22.12) is also able to describe insulin secretion during i.v. tests characterized by smooth glucose profiles such as up and down glucose infusions [39] and hyperglycaemic clamp [37].

Compartment Models

Compartment models are widely used in the biological sciences. They are based on a simple principle of conservation of mass. It was made popular in the 1970s by the book Meadows DH, Randers J, Meadows DL (1972) *Limits to Growth*. Universe Books, New York. A simple example will illustrate the use of the basic principle. Consider a bathtub and the volume of water it contains. We denote this volume by $V(t)$, where we have indicated that this is a function of time. Note that this is proportional to the mass for incompressible water. If we pour water into the tub there is an inflow of water with a rate, say $J_i(t)$. Similarly the outflow of water through the bathtub sink is $J_o(t)$. Now in a small amount of time Δt the change in volume is

$$\Delta V(t) = (J_i(t) - J_o(t)) \Delta t.$$

Passing to the limit $\Delta t \rightarrow 0$ we obtain the differential equation

$$dV(t)/dt = J_i(t) - J_o(t).$$

The inflow may come from a rainwater butt, in which case $J_i(t)$ is the outflow of the rainwater butt. Similarly, the outflow may be to a wastewater tank, in which case $J_o(t)$ is the inflow to the wastewater tank. In this way a system with three compartments, i.e. the rainwater butt, the bathtub and the wastewater tank, is built.

In the present chapter the conserved quantity in the cellular model is the amount of insulin in the granules. Typical compartments are the docked pool and the ready releasable pool. Although these compartments are not physically separated from the rest of the cell environment, there is growing experimental evidence that the separation into these compartments is meaningful. In the minimal models typical compartments are compartment 1: blood plasma and rapidly equilibrating tissue and compartment 2: tissues in slow exchange with plasma, and the conserved quantity is the peptide masses.

Further Reading:

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 Godfrey K (1983) *Compartmental Models and their Application*. Academic Press, London, UK
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Added by the editors

22.4 Minimal Models of Insulin Action and Hepatic Insulin Extraction

Due to the feedback nature of the glucose–insulin system, β -cell function is not sufficient to evaluate the efficiency of glucose homeostasis in a given individual. To this purpose, β -cell function needs to be interpreted in light of the prevailing

insulin action, measured by insulin sensitivity [3], as formulated by the disposition index paradigm [4], which assumes that glucose tolerance of an individual is related to the product of β -cell function and insulin sensitivity. Thanks to its intuitive and reasonable grounds, this measure of β -cell functionality, which was first introduced for IVGTT, has become the method of choice also with other tests, like clamp and OGTT, as reviewed in [15] where some recent developments related to formulation and practical implementation of the disposition index are also discussed.

Since the effect of insulin on peripheral tissues is determined not only by the biologic effect of insulin but also by the amount of insulin to which the tissue is exposed, hepatic insulin extraction provides the third dimension to the metabolic status of an individual. Not only β -cell function but also insulin sensitivity and hepatic insulin extraction can be measured at whole-body level, during an IVGTT or oral tests, by employing glucose [4, 18] and insulin [12, 40] minimal models.

Minimal models of insulin secretion, action and hepatic extraction have been used in a number of pathophysiological studies, including the effect of age and gender on glucose metabolism [2], the effect of anti-aging drugs [27], the influence of ethnicity [35], insulin sensitivity and β -cell function in non-diabetic [38] and obese [11] adolescents, the pathogenesis of pre-diabetes [7, 8] and type 2 diabetes [1, 17].

22.5 Cellular Model of Insulin Secretion

In relation to the clinical interest described in the previous sections, and considering that the physiological task of the β -cell is to secrete insulin, it might be surprising how little work there has been done on modelling insulin secretion, compared to the focus on other aspects of β -cell physiology such as bursting electrical activity and oscillatory calcium levels and insulin secretion, where mathematical modelling has contributed significantly to the understanding of the generation of these rhythmic patterns (for reviews, see [5, 31]).

However, already in the 1970s, Grodsky [22], Cerasi et al. [13] and others did model the pancreatic insulin response to various kinds of glucose stimuli. Due to the limited knowledge of the β -cell biology at that time, these models were phenomenological. Only recently has our knowledge of the control of the movement and fusion of insulin granules increased to a level where we have started to formulate mechanistically based models.

Grodsky [22] proposed that insulin was located in “packets”, plausibly the insulin-containing granules, but also possibly entire β -cells. Some of the insulin was stored in a reserve pool, while other insulin packets were located in a labile pool, ready for release in response to glucose. The labile pool is responsible for the first phase of insulin secretion [22], while the reserve pool is responsible for creating a sustained second phase. This basic distinction has been at least partly confirmed when the packets are identified with granules [20, 30]. Grodsky [22]

moreover assumed that the labile pool is heterogeneous in the sense that the packets in the pool have different thresholds with respect to glucose beyond which they release their content. This assumption was necessary for explaining the so-called staircase experiment, where the glucose concentration was stepped up, each step giving rise to a peak of insulin. There has been no support of granules having different thresholds [28], but already Grodsky [22] mentioned that cells apparently have different thresholds based on electrophysiological measurements. Later, Jonkers and Henquin [25] showed that the number of active cells is a sigmoidal function of the glucose concentration, as assumed by Grodsky [22] for the threshold distribution.

Recently, we have showed how to unify the threshold distribution for cells with the pool description for granules [32], thus providing an updated version of Grodsky's model, which takes into account more of the recent knowledge of β -cell biology. An overview of the model is given in Fig. 22.4.

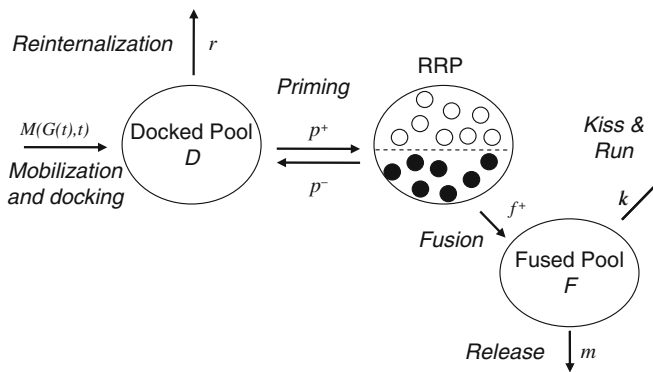


Fig. 22.4 Schematic representation of the mechanistic model [32]. The RRP has been divided into readily releasable granules located in silent cells with no calcium influx, exocytosis and release (*open circles*) and readily releasable granules located in triggered cells (*filled circles*).

It includes mobilization of secretory granules from a reserve pool to the cell periphery, where they attach to the plasma membrane (docking). The granules can mature further (priming), thus entering the “readily releasable pool” (RRP). Calcium influx triggers membrane fusion and subsequent insulin release. We included the possibility of so-called kiss-and-run exocytosis, where the fusion pore reseals before the granule cargo is released. The glucose-dependent increase in the number of cells showing a calcium signal [25] was included by distinguishing between readily releasable granules in silent and active cells. Therefore, the RRP is heterogeneous in the sense that only granules residing in cells with a threshold for calcium activity below the ambient glucose concentration are allowed to fuse. Our model thus provides a biologically founded explanation for the heterogeneity assumed by Grodsky [22] and is able to simulate the characteristic biphasic insulin secretion pattern in response to a step in glucose stimulation, as well as the secretory profile of the staircase stimulation protocol.

The equations of the model are as follows:

$$dM(t)/dt = -[M_\infty(G) - M(t)]/\tau, \quad (22.15)$$

$$dD(t)/dt = M(G,t) - r D(t) - p^+ D(t) + p^- \int_0^\infty h(g,t)dg, \quad (22.16)$$

where M is the mobilization flux, τ is a time delay, D is the insulin in the docked pool and r is the rate of reinternalization. The RRP is modelled by a time-varying density function $h(g,t)$ indicating the amount of insulin in the RRP in β -cells with a threshold between g and $g + dg$, described by the equation

$$dh(g,t)/dt = p^+ D(t) \varphi(g) - p^- h(g,t) - f^+ h(g,t) \theta(G - g). \quad (22.17)$$

Here $\theta(G - g)$ is the Heaviside unit step function, which is 1 for $G > g$ and 0 otherwise, indicating that fusion only occurs when the threshold is reached. The priming flux $p^+ D$ distributes among cells according to the fraction of cells with threshold g described by the time-constant function $\varphi(g)$. The secretion rate is expressed as

$$SR(t) = m F(t) + SR_b, \quad (22.18)$$

where SR_b is basal secretion, m is the rate constant of release and F is the size of the fused pool, which follows

$$dF/dt = -(m + k)F + f^+ H(G, t), \quad (22.19)$$

where f^+ is the rate constant of fusion, k is the kiss-and-run rate and $H(G, t) = \int_0^G h(g,t)dg$ represents the amount of insulin in the RRP in cells with a threshold below G . For further details of the model, we refer to the original article [32].

22.6 Cellular Modelling: Insight into Minimal Models

Modelling of intracellular events helps in understanding the role of different mechanisms of insulin secretion, on both cellular and whole-body levels. We have recently shown that the secretion rate SR (22.18) of the cellular, mechanistic model [32] contains implicitly the three main ingredients of the OGTT minimal models: (i) a *static term*, which includes (ii) a *delay* τ due to the delayed refilling of the docked pool D and (iii) a *dynamic term* proportional to dG/dt [34]. The latter *derivative control* is due to the cellular activation thresholds [25].

The comparison of cellular models to minimal models provides insight into the mechanisms underlying the different aspects of the minimal models and in a sense

justifies them by giving a mechanistic underpinning. Model comparison also provides a link between the secretion indices of the minimal model to cell biological events, thus yielding biological meaning to the indices.

Other recent models go into greater details of the regulation and properties of different pools of granules in single cells [6, 14, 33]. Such details allowed connecting recent imaging experiments [29] with granule properties, such as a highly calcium-sensitive pool [43, 44], and the investigation of the so-called amplifying pathway of glucose-stimulated insulin secretion [23]. We note that although these models describe the dynamics and control of the secretory granules in great detail they are unable to reproduce the crucial staircase experiment, because they do not have any threshold distribution in the sense of Grodsky [22] and in contrast to our recent model [32].

22.7 Conclusions

Models of minimal complexity provide simultaneous assessment of β -cell function, hepatic insulin extraction and insulin sensitivity under physiologic conditions using a simple protocol. Minimal model complexity is an essential property, since reliable estimates of model parameters need to be derived from a limited number of data collected from an individual. The amount of information they provide is appealing, since it conveys novel insights regarding the regulation of fasting and postprandial glucose metabolism in diabetic and non-diabetic humans.

However, in addition to simplicity of the minimal models, it is also desirable that they are physiologic, i.e. they are linked to the underlying biology of the insulin-secreting β -cells. We have recently [34] presented a way to make such a connection using a recent model [32] describing intracellular mechanisms. This analysis showed how the three main components of oral minimal secretion models, derivative control, proportional control and delay, are related to subcellular events, thus providing mechanistic underpinning of the assumptions of the minimal models. Such an understanding of the underlying mechanisms can help interpret differences in β -cell sensitivity indices between different populations or patient groups or give insight into the physiologically most important steps regulated by, for example, GLP-1 [19].

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Chapter 23

Geometric and Electromagnetic Aspects of Fusion Pore Making

Darya Apushkinskaya, Evgeny Apushkinsky, Bernhelm Booß-Bavnbek, and Martin Koch

Abstract For regulated exocytosis, we model the morphology and dynamics of the making of the fusion pore or porosome as a cup-shaped lipoprotein structure (a *dimple* or *pit*) on the cytosol side of the plasma membrane. We describe the formation of the dimple by a free boundary problem. We discuss the various forces acting and analyse the magnetic character of the wandering electromagnetic field wave produced by intracellular spatially distributed pulsating (and well-observed) release and binding of Ca^{2+} ions anteceding the bilayer membrane vesicle fusion of exocytosis. Our approach explains the energy efficiency of the dimple formation prior to hemifusion and fusion pore and the observed flickering in secretion. It provides a frame to relate characteristic time length of exocytosis to the frequency, amplitude and direction of propagation of the underlying electromagnetic field wave. We sketch a comprehensive experimental programme to verify – or falsify – our mathematical and physical assumptions and conclusions where conclusive evidence still is missing for pancreatic β -cells.

Keywords Calcium oscillations · Dimple formation · Free boundary problems · Fusion pore · Lorentz force · Maxwell equations · Pancreatic β -cell · Plasma membrane · Regulated exocytosis

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23.1 Introduction

23.1.1 *On Our Heuristic (Suggestive) Use of Mathematical Modelling*

This chapter adds a few electromagnetic facts and mathematical theorems to the toolbox approaching the process of bilayer membrane vesicle fusion. We address the related geometric and dynamic aspects of the endocytotic–exocytotic cycle which is at the core of various discharge (e.g. secretion) and ingestion (e.g. drug intake) processes in animal cells.

We begin with a *caveat*. From mathematical physics, quantum chemistry and various fields of engineering design we are accustomed to perfect reliability of theoretical calculations due to full understanding of the governing laws and full practical control of the calculated processes. We have learnt, sometimes the hard way, from physics history that, when in doubt, we had better trust the theory and carefully designed elaborate experiments than first views and ad hoc explanations.

Clearly, the situation is different in theoretical biology and medicine. There, it seems to us, the main use of mathematical modelling is either falsification or extrapolation. By *falsification*, we mean the use of simple arithmetic or other more advanced mathematical means to check and falsify common belief (like Harvey's mathematical microscope, see Ottesen [Chapter 6](#), or the harmonic analysis of Ca^{2+} oscillations in Fridlyand and Philipson [Chapter 21](#)). By *extrapolation* we mean the packing of established phenomenology into a precise, intentionally simplified mathematical framework admitting series of computer simulations or analytical estimates to investigate the role of selected parameters (like the Silicon Cell, see Westerhoff et al. [Chapter 19](#), the mesoscopic simulation of membrane-associated processes, see Shillcock [Chapter 20](#)), or the compartment models for different pools of insulin granules in exocytosis preparations, see Toffolo et al. [Chapter 22](#)).

In this chapter, our approach is different. We simply ask (1) Could it be that a highly *localizable* phenomenon like the lipid bilayer fusion of regulated exocytosis on a characteristic length scale of tens of nanometres has essential *cell–global* aspects on a characteristic length scale of hundreds and thousands of nanometres? (2) Could it be that the observed changes of the electrostatic plasma membrane potential accompanying regulated exocytosis and the corresponding Ca^{2+} oscillations have an electro-magnetic character which requires a field-theoretic (Maxwell) approach to the secretion process?

We have good reason for our at present still speculative but hopefully suggestive approach, both in the re-interpretation of more or less well-observed phenomena and in focusing on aspects which seem to us not sufficiently supported by common explanations. This will be explained below.

We shall emphasize that the correctness of the electro-dynamical and mathematical modelling parts of the findings of this chapter depends on future experimental testing and biological validation. At the end of this report, the reader can find a comprehensive list of experiments that really need to be done to confirm the relevance of

our physical equations and mathematical modelling. Therefore, our models do not aim for instant clarification but rather set a scene for alternative considerations and future observations. That is what we understand by the *heuristic* (suggestive) use of mathematics.

23.1.2 Electromagnetic Free Boundary Route to Fusion Pore Making

For regulated exocytosis, we model the morphology and dynamics of the making of the fusion pore or porosome as a cup-shaped lipoprotein structure (a *dimple* or *pit*) on the cytosol side of the plasma membrane. One ingredient to our model is a free boundary problem for the dimple under the action of electromagnetic forces, in particular the Lorentz force acting on charged molecules of the cell's plasma membrane with decreasing capacitive reactances while forming the dimple. The force comes from a wandering electromagnetic field produced by intracellular spatially distributed pulsating (and well-observed) release and binding of Ca^{2+} ions.

Our approach is based on variational principles and emphasizes regularity and singularity under the deformation process of the membranes. It explains the energy efficiency of the observed dimple forming prior to hemifusion and fusion pore and the observed flickering in secretion. It provides a frame to relate characteristic time length of exocytosis (ranging between milliseconds in nerve cells and seconds in β -cells) to the frequency, amplitude and direction of propagation of the underlying electromagnetic field wave.

We shall not address all the *machines* (both protein machines and lipid assemblies) working together in making the structure and the composition of the fusion pore. Admittedly, conclusive evidence is still lacking of the critical character of the here described electromagnetic field wave for the well-functioning of the regulated exocytosis in healthy cells and the lack of secretion robustness in stressed cells. However, the present electromagnetic free boundary model gives various hints to future calculations, estimates, and in vivo, in vitro and in *silico* (i.e. numerical simulation) experiments.

23.1.3 Plan of the Chapter

In Section 23.2 we summarize several mathematical, electrodynamical and cell physiological facts which seemingly have been overlooked or discarded in the literature, but may in our perception add essential ingredients to a comprehensive understanding of the short very first phase of regulated exocytosis. In Section 23.3 we describe our model and the corresponding differential equations, force balances and cost functionals. In Section 23.4 we discuss regularity and singularity results. In Section 23.5 we present our preliminary conclusion, some hints regarding the

question of what controls the speed of the process and a review of experimental tasks and capabilities to test our hypotheses.

23.2 Synopsis of Established Facts

We describe the challenge of making the fusion pore; distinguish different mathematical modelling approaches; and elaborate electromagnetic and geometric phenomena of the very first phase of regulated exocytosis, namely Ca^{2+} oscillations, the corresponding slow and low frequent electromagnetic field wave and the forming of a dimple in the plasma membrane prior to the bilayer membrane vesicle fusion.

23.2.1 Membrane Fusion and the Fusion Pore Challenge

In animal cells, membrane fusion between the plasma membrane and transport vesicles is fundamental for the secretion of macromolecules. In contrast, the opposite event, i.e. the forming of vesicles or endosomes from the plasma membrane, is necessary for the uptake of macromolecules and nutrients. The latter process is known as *endocytosis*, in which, in the suggestive words of a renown textbook, “localized regions of the plasma membrane invaginate and pinch off to form endocytotic vesicles” (Alberts et al. [1]). The process of discharge of material, after collecting it in transport vesicles, is called *exocytosis* and is our subject.

It happens by docking of the vesicle to the cell membrane through activity of several membrane-associated proteins, followed by vesicle membrane hemifusion and the making of a fusion pore in the membrane through which the material can be released to the exterior, see Fig. 23.1. As a rule, the two processes, i.e. endocytosis and exocytosis, seem to be balanced, the one cutting membrane pieces out of the cell membrane, the other inserting pieces. In both processes, the remarkable is the opening of the cell *without* pinching a hole.

Typical examples of endocytosis are the intake of nutrition, signal molecules, viruses or drugs; typical examples of exocytosis are secretion of macromolecules such as hormones from endocrine cells, inflammatory mediators from immune cells, neurotransmitter release from nerve cells or the removal of waste molecules and bi-products. The β -cells located in the islets of Langerhans in the pancreas are strongly secretory active in producing and releasing insulin. It seems that a better understanding of these two processes could, e.g., support the early diagnosis of metabolic diseases, including diabetes mellitus type 2 (exocytic dysfunction, see Rorsman and Renström [4, 5]), or a more efficient delivery of insulin analogues (inducing endocytosis).

Recent advances in observational and manipulative nanotechniques and in mesoscopic coarse-grained computer simulation have provided substantial progress in visualizing, understanding and – possibly – influencing the bilayer membrane vesicle fusion (for a recent systematic review we refer to Shillcock and Lipowski

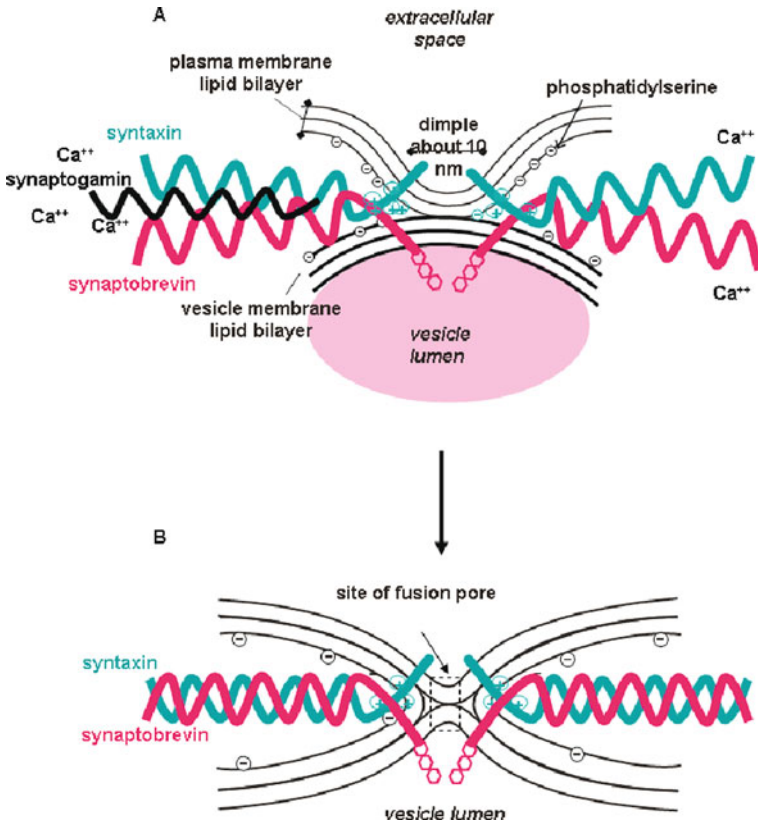


Fig. 23.1 Schematic view of the bilayer membrane fusion event, from Koch et al. [2] (after Lentz [3]), reproduced with permission of World Scientific Publishers and the authors. Shown are the basic molecular structures involved in the process of fusion pore making and membrane fusion. Note the lipid bilayers of the plasma membrane and the vesicle membrane, with the negative charges representing the charged heads of phosphatidyl serine. For clarity, only the transmembrane proteins syntaxin and synaptobrevin with the three aromatic amino acids on the luminal side of the vesicle membrane are depicted. Also included is synaptogamin, which binds Ca^{2+} and is associated with both syntaxin and synaptobrevin.

[6]). With present technology, however, the observational findings are hampered: geometric shape *and* the parameters of change cannot be measured to the wanted degree of precision, *simultaneously*. So Le Bris [6, p. S1196], deplors that “the molecular rearrangements that take place during the final stage of the fusion process, where the two initially distinct membranes join and produce a fusion pore, cannot yet be resolved by these experimental techniques” while “understanding how the stability of lipid membranes is overcome by the cellular protein machinery when required is a major topic of research” (l.c.). This is the challenge which we wish to address by summarizing various mathematical, electrodynamic and empirical facts. Seemingly, these facts have been overlooked or discarded in the literature.

We shall show, however, to what extent these facts yield, in our perception essential, ingredients to a comprehensive understanding of this short very first phase of regulated exocytosis.

23.2.2 *Competing Mathematical Approaches to Space–Time Processes*

We shall distinguish three different mathematical–numerical approaches to modelling spatial–temporal process of regulated exocytosis: highly aggregated compartment models, spatially distributed dynamical systems and space–time integrating partial differential equations, where our focus will be. Clearly, all three approaches admit extensions from rigid, stiff and hence fragile deterministic to more robust stochastic modelling. Here, however, we shall not discuss such extensions.

A first class of mathematical exocytosis models is *Compartment Models*, first introduced by Grodsky [7] in 1972, assuming that there are two compartments (pools) of insulin granules, docked granules ready for secretion and reserve granules. By assuming suitable flow rates for outflow from the docked pool and resupply from the reserve pool to the docked pool, the established biphasic secretion process of healthy β -cells could be modelled *qualitatively* correct. By extending the number of pools from two to an array of six and properly calibrating all flow rates, Chen et al. [8] obtained a striking *quantitative* coincidence with the observed biphasic process, see also Toffolo et al. [Chapter 22]. The nice thing by such compartment models is that they invite the experimentalists (both in imaging and in proteomics) to verify the distinction of all the hypothetical compartments in cell reality and to assign biophysical values to the until now only tuned flow rates. A self-imposed limitation is the low resolution of the aggregated compartments which does not allow to investigate the local geometry and the energy balance of the secretion process.

On the opposite length and time scale, we have a second class of mathematical models, namely the impressive numerical analysis of the bilayer membrane vesicle fusion by *Molecular Dynamics* (MD), *Monte Carlo simulations* (MC) and *Dissipative Particle Dynamics* (DPD) on nanometre distances and fractions of nanoseconds, based on gravitational and electric forces between the particles, see the afore-cited [6] and Shillcock, Chapter 20. Unfortunately, these computer simulations are also seriously hampered, namely, by limitations of present hardware and software when one is addressing mesoscopic behaviour, i.e. changes across many scales of the molecular characteristics – in spite of the impressive results when applying these methods to phenomena on the nanoscale, like modelling the island dynamics of film crystallization in epitaxial growth driven by molecular beam epitaxy, see, e.g. Caffisch and Li [9]. These limitations in present computer capacity require MD, MC and DPD simulations to make a priori assumptions about the *pathway* of the fusion process, e.g. spherical symmetry of the vesicles and planarity and circularity of the fusion pores – besides the often deplored “enormous gap between the sophistication of the models and the success of the numerical approaches used in practice and, on the other hand, the state of the art of their rigorous understanding”

(Le Bris [10] in his 2006 report to the International Congress of Mathematicians). To keep these models transparent, a self-imposed limitation is their focus on the local neighbourhood of the fusion event, neglecting long-distance phenomena like electromagnetic waves across the cell.

Whereas compartment models, respectively, MD and DPD are built upon small, respectively, huge systems of ordinary differential equations with each unknown specifying temporal changes in one given pool or spatial box, we advocate a third class of mathematical models, namely modelling the dynamics and the geometry by *partial differential equations*. Consequently, we shall try to model the relevant processes by one or two spatial–temporal equations based on First Principles, instead of the few aggregated purely temporal pool equations in compartment modelling or the three millions of purely temporal equations for spatially distributed boxes in [6, p. 1197] (which still is a very poor particle number for a $100\text{ nm} \times 100\text{ nm} \times 42\text{ nm}$ simulation box).

Moreover, the simplicity of our fundamental equations admits a transparent incorporation of long-distance phenomena. In such a way, our approach takes its point of departure not only in the rather well-studied *elastic* and *electric* properties and potentials of and across the plasma membrane and the *viscosity* of the cytosol, but in non-stationary, dynamic *electromagnetic* properties. To us, the basic electromagnetic character of the fusion process becomes evident in

- the observed electromagnetic (wandering) field waves, see Section 23.2.3,
- the closure of the corresponding magnetic wave over the plasma membrane, see Section 23.2.4,
- the observed forming of a narrow dimple, solely to be understood like a capacitor, see Section 23.2.5 and
- the observed flickering of the secretion process corresponding to the natural variability of the (wandering) field wave generation, see Section 23.2.6.

The goal of our approach is

1. to develop a simple free boundary model for the dimple forming process, see Section 23.3,
2. to focus on regularity and possible singularity of the free boundary, see Section 23.4,
3. to provide a reliable framework for estimating (and, hopefully, influencing) the parameters which control the speed of the process, see Section 23.5.2
4. and to formulate a bundle of model-based observation plans to verify or falsify our assumptions, see Section 23.5.3.

Our approach is inspired by recent work of Friedman and collaborators about tumour growth, see [11–14]; by a theoretical analysis of adhering lipid vesicles with free edges, see [15] by Ni et al.; by the electrodynamic challenge to understand the observed Ca^{2+} oscillations, rightly perceived as being “contradictory and often do not support the existing (electrostatic) models” (Fridlyand and Philipson

[Chapter 21](#)) and by the mathematical challenge to understand the (in elastic terms counter-intuitive) dimple formation so well described in the literature, see, e.g. Monck and Fernandez [18], Rosenheck [19], Lentz et al. [3], Koch et al. [2].

23.2.3 Oscillatory Intracellular Release and Binding of Ca^{2+} Ions

We recall a few basic observations of Ca^{2+} oscillations and postulate a simple but powerful procedure of generating an electrodynamic field.

23.2.3.1 Basic Observations of Ca^{2+} oscillations

By fluorescence microscopy, empirical evidence has been provided about pulsating Ca^{2+} activity at extreme low frequency $f \sim 0.1 \text{ Hz} \ll 3 \text{ Hz}$ (for comparison, the house low frequency grid is of 50 Hz, i.e. spikes in intervals of 20 ms), prior to the fusion event, see Kraus et al. [20] and Bernhard Wolf's homepage [21] with informative video animations of calcium oscillations and the comprehensive review and analysis by Salazar et al. [22].¹ Wolf's observations were made with HELA cancer cells. Höfer et al. give a general model, based mainly on observations in muscle cells.

For corresponding observations for β -cells, we refer to Maechler, [Chapter 3](#), and Fridlyand and Philipson, [Chapter 21](#), in this volume, who deal with various types of low-frequent oscillations.

The following can be seen in many cell types: when a cell is stimulated ("polluted") by a Ca^{2+} -mobilizing stimulus, the changes in the cytosolic calcium concentration $[\text{Ca}^{2+}]_c$ occur as repetitive spikes that increase their frequency with the strength of the stimulus (see also Berridge et al. [24] and Gaspers and Thomas [25]). It is well known that an increase in $[\text{Ca}^{2+}]_c$, ultimately, regulates a plethora of cellular processes mediated by Ca^{2+} -dependent enzymes that, in turn, modify downstream targets commonly by phosphorylation.

Investigating Ca^{2+} decoding in an analytically tractable generally applicable model, Höfer and collaborators address the question "Under which conditions are Ca^{2+} oscillations more potent than a constant signal in activating a target protein?" in [22, p. 1204] in complex biochemical terms of binding and release rates of Ca^{2+} ions.

¹Our model cell is a pancreatic β -cell where a single release is slow and may take seconds. Correspondingly, we expect a low Ca^{2+} oscillation rate in intervals in the range of seconds yielding extreme low frequency of the observed 0.1 Hz. For nerve cells, the reaction time, and so the release time, is in the range of milliseconds, possibly less than 100 μs , see Jahn et al. [23]. Correspondingly, we expect a high Ca^{2+} firing rate in intervals of, e.g. 10 ms yielding a frequency of 100 Hz with associated high energy losses. So, our electromagnetic free boundary route to vesicle fusion cannot function in nerve cells unless the neurotransmitter vesicles are kept waiting very close to the plasma membrane. Here our finding coincides with the well-known deviating high-energy consumption of nerve cells. We shall elaborate this aspect in [Section 23.5](#).

23.2.3.2 Postulated Electrodynamic Field Character

We wish to supplement these investigations by recalling pieces of circumstantial evidence which may support the hypothetical electromagnetic character of the Ca^{2+} oscillations. As said before, Ca^{2+} handling is extremely complex.

1. There are various Ca^{2+} -storage organelles in the β -cells, first of all the spatially distributed and clearly separated mitochondria and the spatially rather extended smooth endoplasmic reticulum – SER, see also (Chapter 21, Section 5).
2. There is some agreement in the literature that the handling by the mitochondria, is decisive for, at least, the slow Ca^{2+} oscillations: “Metabolic profiling of β -cell function identified mitochondria as sensors and generators of metabolic signals controlling insulin secretion”, according to Maechler (Chapter 3); and Fridlyand and Philipson (Chapter 21) refer to “data demonstrating that slow oscillations can persist in the presence of thapsigargin, the agent that blocks SERCA and empties the ER stores...”.
3. To us, these observations fit nicely with our postulate of the electrodynamic character of the slow Ca^{2+} bursts: spatially distributed and temporarily coordinated (not necessarily simultaneous) release and uptake of Ca^{2+} ions can generate an electrodynamic field with magnetic character.

Of course, there are many mechanisms and systems that contribute to the release and uptake of Ca^{2+} from intracellular organelles, some of these even have bell-shaped effects on Ca^{2+} release, and phenomena such as Ca^{2+} -induced Ca^{2+} release also take place (these facts were communicated to the authors by Pociot and Størling [16]). In the following, we anticipate the existence of a (not yet fully confirmed) system of aggregated build-up of an electrodynamic field with magnetic character in β -cells upon glucose stimulus: In short, we have movements of Ca^{2+} ions in and out of the mitochondria. Movements of ions are currents. In-and-out movements are AC currents. AC currents produce AC fields or, rather, AC field oscillations. Superposed oscillations may produce moving fields.

In this way, we suppose the organelles build an alternating electric current density (also “displacement vector field”) $\mathbf{D}$ of low frequency by superposition of spatially distributed, temporally coordinated and directed Ca^{2+} activity. As usual in electrodynamics, we shall speak of two different electric fields, $\mathbf{D}$ and $\mathbf{E}$. This second electric field $\mathbf{E}$ is given by the relation $\mathbf{D} = \varepsilon \mathbf{E}$, where $\varepsilon = \varepsilon_0 \varepsilon_r$ denotes the dielectric constant. Note that our writing of all electromagnetic units and equations follows Jackson [17] in the units V, A, s and m with, e.g. $\text{kg} = \text{VAs}^3/\text{m}^2$ of the System International – SI, which is predominant in engineering literature.

A physical model of generating electromagnetic field waves by spatially and temporally distributed excitation was built by Koch and Stetter, see <http://www.feldkraft.de/>. It is called *Dynamical Marker* and consists of a couple of coil arrays, electronically regulated for direction-, frequency- and amplitude-controlled generation of a field wave. The instrument has been applied in various

cell physiological experiments for slow and efficient transport of beads into cells across the plasma membrane.

There are a couple of related questions which will require separate investigation (see also Section 23.5.3):

1. How strong is the evidence that the Ca^{2+} *oscillations*, contrary to the membrane process of Ca^{2+} bursts, originate from an array of Ca^{2+} depots (SER and mitochondria), organized in directed lines?
2. How does the cell select the Ca^{2+} storages to participate in the generation of the alternating current?
3. How is the sequential release and binding of the Ca^{2+} ions of the different storages controlled, i.e. how is the correct spatial and temporal coordination of release and binding obtained?
4. What role plays the observed branching of mitochondria in active β -cells, contrary to the dipole shape of mitochondria in tired β -cells (see Fig. 23.2)?
5. Can the magnetic character of the field wave, which is produced by low frequent Ca^{2+} oscillations, be influenced via an external field with similar character?

To questions 1 and 2, it may be mandatory to distinguish between two different types of Ca^{2+} burst: the Ca^{2+} *oscillations* addressed here are prior to the secretion and are, presumably, generated by arrays of activated calcium depots distributed through the full length of the cell. On the contrary, the Ca^{2+} *influx* through the ion channels of the plasma membrane during regulated exocytosis is mainly effective

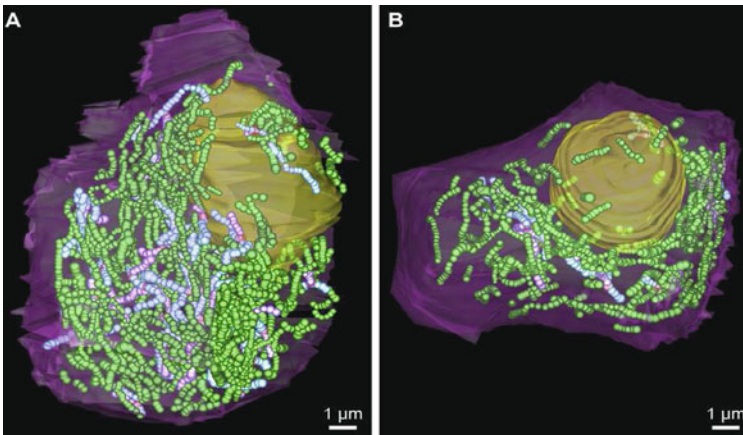


Fig. 23.2 Functional heterogeneity: *left*, a multitude of *branched* mitochondria in a vigorously responding β -cell; *right*, relatively more non-branched *dipole* mitochondria in a less active cell at a comparable locus; branch points are highlighted with *red spheres*. The panels are from Noske et al. [26] and reproduced with permission of Elsevier. High-resolution originals were courtesy of B. Marsh, University of Queensland, Brisbane, Australia.

close to the plasma membrane where it increases the concentration $[Ca^{2+}]_c$ and changes the electric potential across the plasma membrane. It is worth mentioning that the array character is evident from the observed oscillations in the form of *directed, oriented waves*.

To question 3, we imagine that the coordination of the activity of the participating Ca^{2+} storages is not controlled externally, e.g. by the nucleus, but happens spontaneously by self-organization: we notice that the storages of molecular calcium sense and respond to stimuli by periodic release and binding of Ca^{2+} ions and suppose that the sequential coordination between spatially distributed loci of release and binding minimizes the energy consumption for maintaining the activity and for establishing a suitable average concentration $[Ca^{2+}]_c$.

To question 4, we recall from Noske et al. [26] a remarkable discovery. The authors imaged and reconstructed two β -cells from the same glucose-stimulated mouse islet by single axis, serial section electron microscope tomography (ET) at magnifications of $4700\times$ and $3900\times$, respectively, that resulted in whole cell tomograms with a final resolution of 15–20 nm. In addition, they developed several new methods for the abbreviated segmentation of both cells' full complement of mitochondria (i.e. the most prominent Ca storages) and insulin secretory granules for comparative analysis. Three-dimensional reconstruction by ET of each of the two β -cells (designated 'ribbon01' and 'ribbon02') indicated that ribbon01 responded more vigorously to glucose stimulation than ribbon02 and contained about twice as many branched mitochondria (26 out of a total number of 249 mitochondria) as ribbon02 (with 10 branched mitochondria out of a total of 168 mitochondria). See also the recent Marsh and Noske, Chapter 8, in this volume.

Now, from our electromagnetic point of view, the advantage of branched mitochondria is clear for the generation of a field wave (i.e. the pulsating Ca^{2+} oscillations through the whole length of the cell): a single (nonrotating) dipole cannot generate or initiate a (directed, oriented) field wave. That *requires* a branched structure with spatially and temporally shifted serial activity, as demonstrated also in the design of the mentioned simple field generator by Koch and Stetter. Of course, nature's regulation of the ion firing may be much more sophisticated than the crude engineering design of the "Dynamic Marker". After all, the eucaryotic cells had many more years to test and optimize different designs in evolution.

To question 5, we refer to an experimental setting described in Section 23.5.3.

23.2.4 The Magnetic Character of the Induced Field Wave

Perhaps one of the most delicate of Maxwell's equations (cf. Box) is his modification of Ampère's law by adding the displacement current density $\frac{\partial \mathbf{D}}{\partial t}$ on the right, i.e. the electric side:

$$\text{curl } \mathbf{H} = \mathbf{J} + \frac{\partial \mathbf{D}}{\partial t}, \text{ respectively, } \int_C \mathbf{H} d\mathbf{s} = \int \int_A \left(\mathbf{J} + \frac{\partial \mathbf{D}}{\partial t} \right) d\mathbf{A}. \quad (23.1)$$

Here $\mathbf{H}$ denotes the oriented, electrically generated magnetic field, $\mathbf{B} = \mu_0\mu_r\mathbf{H}$ the corresponding magnetic flux density (also “magnetic induction”) of frequency f and amplitude $\hat{\mathbf{B}}$ and $\mathbf{J}$ the current density vector for a conductor crossing an area A which is bounded by a contour C . Moreover μ_0 and μ_r denote the absolute and relative magnetic permeabilities.

The Four Maxwell’s Equations at a Glance

Gauss’ law $\nabla \cdot \mathbf{D} = \rho_f$ describes how an electric field is generated by electric charges: There is a physical quantity, called charge. This quantity is the source of electric fields. Spatial differences in an electric field originate from the charge density.

Gauss’ law for magnetism $\nabla \cdot \mathbf{B} = 0$, i.e., there are no magnetic quantities, comparable to charges, which lead to spatial changes in the magnetic field.

Faraday’s law $\nabla \times \mathbf{E} = -\frac{\partial \mathbf{B}}{\partial t}$ describes how a changing magnetic field can create (*induce*) an electric field.

Ampère’s law with Maxwell’s correction $\nabla \times \mathbf{H} = \mathbf{J}_f + \frac{\partial \mathbf{D}}{\partial t}$ states that magnetic fields can be generated in two ways: by electrical current (this was the original *Ampère’s law*) and by changing electric fields (this was *Maxwell’s correction*).

Maxwell’s illustrious contribution was to combine the four laws into one single mathematically aesthetic and operational and physically instructive system of differential equations.

$\mathbf{E}$ is the *electric force field*, measured in Volt per metre, and

$\mathbf{D}$ is the *electric flux field* or displacement field, also called the electric induction or the electric flux density. It is measured in Coulombs per square metre.

$\mathbf{H}$ is the *magnetic force field* or magnetizing field, also called the auxiliary magnetic field, the magnetic field intensity or just the magnetic field. It is measured in Ampères per metre, and

$\mathbf{B}$ is the *magnetic flux field*, also called the magnetic induction, the magnetic field density or the magnetic flux density. It is measured in Tesla.

ρ_f is the *free charge density*, measured in Coulombs per cubic metre.

$\mathbf{J}_f$ is the *free current density*, measured in Ampères per square metre.

Further Reading:

Jackson, J.D. (1999) Classical electrodynamics, 3rd ed. John Wiley, New York, NY

Added by the editors

Equation (23.1) is our basic equation regarding the magnetic character of the observed Ca^{2+} oscillations. Note that our field wave cannot be compared with an electromagnetic high frequency wave in radio transmission. Its propagation velocity is comparable to sound waves in water and far below light velocity. Moreover,

because of the low frequency of our oscillations, the displacement current density $\frac{\partial \mathbf{D}}{\partial t}$ is relatively small. That indicates that the magnetic character dominates the electric character of the field wave.

While a wave with dominant electric character has large losses in cytosol (which is comparable to salty water in its electric conductivity), the magnetic character makes the propagation almost free of losses. To sum up, by the described intracellular Ca^{2+} oscillations a field is generated with only marginal losses because the transmission is almost independent of the material constant ε_r . As a result, the moving Ca^{2+} ions from the intracellular distributed storages are in fact an AC current, generating a nearly loss-free moving (wandering) magnetic *field wave* which transfers energy to a selected transmembrane subregion of the cytosol between the plasma membrane and a single vesicle. As we shall explain, this energy and the corresponding forces act on the free ions distributed in one or other way among the phospholipids of the plasma membrane and pull the plasma membrane towards the vesicle. See also [27] for a video animation of a macroscopic field wave.

Our biophysical approach is classical, hence we assume that there are no sources for magnetism, no magnetic monopoles, at least not present in our β -cell. Consequently, we obtain from Maxwell's equations $\text{div} \mathbf{B} = 0$, i.e. the magnetic field wave induced by the alternating current is *closed* in the sense of vector analysis and, consequently, the path of the field wave is closed.

Of course, it must be investigated in detail *how* the magnetic wave is closed. Fluorescence microscopy gives the impression that the observed Ca^{2+} oscillations are collective phenomena of cell ensembles: the oscillations propagate through the ensemble like chained waves. We, however, assume that all magnetic waves are separated from each other and are closed over the single plasma membranes. One reason is that the plasma membrane is perforated by a multitude of ion channels created and maintained by the presence of enzymes like various kinases and phospholipases. Most enzymes like the mitochondrial cytochrome contain Fe atoms, see Jensen [28, pp. 134f] and the review on iron biominerals [29]. Consequently, the magnetic field wave will search for a circuit through the plasma membrane.

While we have put the determination of the Fe content of the plasma membrane on our experimental agenda in Section 23.5.3, we should mention that the magnetic permeability μ_r cannot be measured directly. However, there are methods to determine the magnetic permeability $\mu_r \sim 1.0000007$ of haemoglobin of deoxidized venous blood noninvasively. Similar methods will be applicable for investigating the plasma membrane. Roughly speaking, the indirect methods work by comparative measurements after inflicting a magnetic pollution on a harmonic oscillator.

Now it is not difficult to understand the making of the fusion pore and the dimple formation (see Section 23.2.5 for the empirical evidence) in qualitative terms. Let us fix the notation. By electron microscopy, we can distinguish the following clearly separated regions:

- D_0 Amorphous outside cell neighbourhood
- D_1 Plasma membrane with boundary $\partial D_1 = \Gamma_{1-0} \cup \Gamma_{1-2}$
- D_2 Cytosol

D_3	Vesicle membrane with boundary $\partial D_3 = \Gamma_{3-2} \cup \Gamma_{3-4}$
D_4	Vesicle lumen
M_j	Activated molecular Ca storage organelles, $j = 1, \dots, n$ (not depicted)
N	Cell nucleus (not depicted)

Note that the region D_1 (the plasma membrane) consists of a phospholipid bilayer. It has a surface Γ_{1-0} as its outside boundary (towards the amorphous outside cell neighbourhood D_0) and a surface Γ_{1-2} as its inside boundary (towards region D_2 of the watery cytosol within the cell close to the plasma membrane). Moreover, we have the region D_3 consisting of the vesicle membrane (same material like D_1) and the region D_4 , the interior of the vesicle, containing the material to be released through the plasma membrane. Moreover, there are a multitude of activatable Ca^{2+} storage organelles $\{M_j\}$ spread through the interior of the cell. Finally, we have the cell nucleus N , see the abstraction of Fig. 23.1 in Fig. 23.3. Typical approximate diameters are 10 μm for most animal cells, 5 μm for the nucleus, 100 nm for the vesicles and 8 nm for plasma and vesicle membrane.

In the preparation of the fusion event and the making of the fusion pore, there are apparently only two active regions, in addition to the cell nucleus and the mitochondria and other Ca storages, namely the plasma membrane D_1 and the cytosol D_2 . The plasma membrane D_1 forms a conical inside oriented *dimple* (pit) towards the vesicle of around 10 nm base diameter and 10–20 nm height. For the true lipid bilayer membrane–vesicle fusion event, following the making of the dimple, so-called transmembrane proteins become active in the cytosol region D_2 between plasma membrane dimple and the vesicle and pull and dock the vesicle membrane D_3 to the plasma membrane D_1 over a distance of up to 100 nm (see also Fig. 23.1).

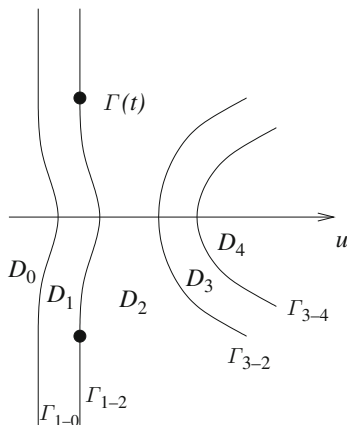


Fig. 23.3 Plasma membrane section with cone-like dimple (*left*) under transversal displacement u and spherical vesicle (*right*) before docking. For a regular cone-shaped dimple, the two **bold dots** mark the circular base line $\Gamma(t) = \partial\{(x, y, z) \mid u(x, y, z, t) \geq 0\}$ of the dimple at time t . In space-time, the union $\{\Gamma(t) \times \{t\}\}_{t \geq 0}$ of the t -components $\Gamma(t)$ forms the free boundary Γ , see Section 23.4.1 before Eq. (23.16) and Section 23.4.4.

When the deformation of the plasma membrane is sufficiently sharp it ends in a branch point, i.e. a singularity of Γ_{1-2} , called hemifusion. Then it comes to a breakthrough (called fusion pore), and the content of the vesicle begins to diffuse from the vesicle compartment D_4 into the outside region D_0 . It appears that this process sometimes is interrupted (so-called *flickering*, see below), i.e. the fusion pore is hardly maintained by elastic forces alone but needs probably the presence of an electromagnetic field and is interrupted when this magnetic field is interrupted.

What is controlling the well-functioning of the fusion event?

Working hypothesis 1: The regions

$$D_0 (X_C = 0), D_1 (\text{variable } X_C(x, t)) \quad \text{and} \quad D_2 (X_C = 0) \quad (23.2)$$

are distinguished by their *capacitive reactance* $X_C := 1/(\omega C)$ where $\omega = 2\pi f$ with constant f and C denotes the capacitance. Note that forming the dimple produces an increase of the dielectricum (between the “plates”) and so implies increasing C and decreasing X_C until X_C vanishes in the fusion pore.

Working hypothesis 2: The vesicle is densely packed with material and so not subjected to deformations easily.

Working hypothesis 3: We envisage the following feedback mechanism for forming the dimple and preparing the fusion event.

1. The Ca^{2+} ions from locally distributed intracellular Ca molecule storages start low frequent oscillations, as described in [20], which are superposed in a controlled way, and a dynamic field wave is produced pointing to a specific region $D_{2,\text{crit}}$ selected for most suitable membrane-vesicle fusion. In the beginning, the magnetic flux density vector $\mathbf{B}$ (“magnetic induction”) is low because the magnetic wave does not easily enter the plasma membrane D_1 to close itself in a circuit because of the originally high X_C in D_1 .
2. The form change decreases X_C close to the emerging dimple. That permits the magnetic wandering wave to enter D_1 more easily and so increases its current density (the sharpness of its pointing) and its amplitude $\hat{B}$. And so on.

23.2.5 Dimple Formation Prior to the Fusion Event

In the introduction to this chapter we defined the fusion pore as the molecular structure that transiently connects the lumens of two membrane compartments during their fusion. We emphasized that making the fusion pore plays a key role in all intracellular trafficking and endocytotic and exocytotic pathways in all eucaryotic cells, including the regulated exocytosis in endocrine, exocrine and neuronal cells like our β -cell. However, from Monck and Fernandez [18, 1992] to Shillcock and Lipowski [6, 2006], researchers agreed that *despite its importance, the nature of the fusion pore is unknown* ([18, p. 1395]).

In a remarkable series of micrographs, based on rapid freezing techniques for electron microscopy, the renowned expert on mammal egg cells, Douglas Chandler from Arizona State University, and collaborators captured the formation of the fusion pore in mast cells already some 30 years ago [30–32]. They demonstrated that the pores are made of a curved bilayer which spans the granule and plasma membrane. The micrographs also gave a hint of the events preceding the making of the fusion pore, namely the formation of a dimple that approaches the granule membrane after stimulation, see Fig. 23.4 and 23.5.

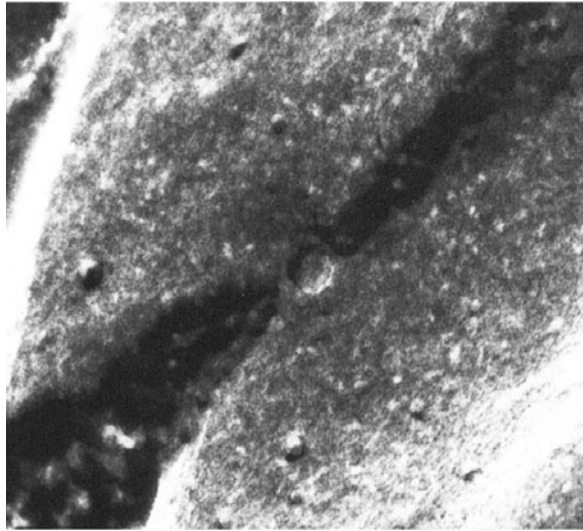
Since then, the dimple formation has been observed in many different cell types immediately upon stimulation before the fusion event, see, e.g. Jena and collaborators [33]. Using atomic force microscopy (AFM) they demonstrated the presence of many simultaneous dimples in pancreatic acinar cells after exposing them to a secretagogue. The paper contains references to analogous demonstrations revealing the presence of pits and depressions also in pituitary and chromaffin cells prior to secretion. To the best of our knowledge, capturing the very fusion event of regulated exocytosis in pancreatic β -cells has not yet been achieved by present imaging methods.

Not surprisingly, the secretory granules have never been seen to form dimples on their own membrane, in accordance with the common perception that the plasma membranes are relatively slack and the membranes of densely packed granule under tension. That corresponds to our preceding Working Hypothesis No. 2.



Fig. 23.4 Cross section through plasma membrane with dimple and, at the bottom, a glimpse of the granule. Electron micrograph courtesy of D. E. Chandler, Arizona State University, Tempe, Arizona.

Fig. 23.5 The fusion pore shown as imprint of the plasma membrane dimple onto the granule membrane, from [32, Fig. 9C]. Reprinted with permission of Springer-Verlag. The high-resolution micrograph was courtesy of D. E. Chandler, Arizona State University, Tempe, Arizona.



23.2.6 The Flickering of Regulated Exocytosis

Another feature of the fusion pore making requires explanation (and is nicely explained by instabilities of the discussed AC current), namely the flickering of the fusion event, i.e. the common observation that the fusion pore can be maintained only after a while of opening and is not stable immediately after its making. Irregular rapid pore openings and closures are observed that last from a few milliseconds to many seconds, see Fernandez et al. [34] for fusion-pore flickering (*kiss-and-run*) in mast cells and Rosenheck [19] and Jahn et al. [23] for wider reports on the observed flickering of the fusion event, mostly for synaptic vesicle exocytosis.

23.3 The Model

In this section we fix our notation and introduce the basic equations for the propagation of the electromagnetic (wandering) field wave and the making of the dimple.

23.3.1 The Force Balance Equation

Let $\mathbf{r} = \mathbf{r}(x, y, z, t)$ denote the displacement vector of the dimple and m the mass of an elementary unit of the dimple. Then the resulting force for making the dimple is approximately equal to $m \partial^2 \mathbf{r} / \partial t^2$, i.e. we can write

$$m \frac{\partial^2 \mathbf{r}}{\partial t^2} = \mathbf{F}_{\text{elas}} + \mathbf{F}_{\text{vis}} + \mathbf{F}_{\text{ext}}, \quad (23.3)$$

where $\mathbf{F}_{\text{elas}}$ denotes the restoring (or elastic) force, $\mathbf{F}_{\text{vis}}$ stands for viscosity reaction of the medium surrounding our plasma membrane and $\mathbf{F}_{\text{ext}}$ is an external force providing the membrane displacement from equilibrium state.

Our first task is to describe the restoring force $\mathbf{F}_{\text{elas}}$. In first approximation, we shall assume that the plasma membrane is a surface in $\mathbb{R}^3$ without bending resistance. Hence $\mathbf{F}_{\text{elas}}$ is defined only by the surface tension and the variation of the membrane surface area.

To describe $\mathbf{F}_{\text{elas}}$ and $\mathbf{F}_{\text{vis}}$ more precisely, we adapt the standard textbook model for the suspended vibrating string, respectively, vibrating plate (see, e.g. Churchill [35, Sect. 93] and Logan [36]). We consider 1D- and 2D membranes separately, because the restoring force behaves differently in one-dimensional and many-dimensional cases. We begin with the 1D case, since all the arguments are simpler in that situation.

23.3.2 The 1D Case

In the equilibrium state, let our 1D membrane coincide with the x -axis; let $u = u(x, t)$ denote the displacement of our plasma membrane from equilibrium state at the point x and at time t ; and let $\rho = \rho(x)$ denote the linear membrane density at the point x . We restrict ourselves to sufficiently small deformations, so, for now, we will neglect all the terms that are of higher infinitesimal order with respect to $\partial u / \partial t$.

Since our plasma membrane has no bending resistance, its tension $\mathbf{T}(x, t)$ at the point x and at time t is directed along the tangent to the membrane at x . Therefore, the unit $(x, x + \Delta x)$ is subjected to tensions $\mathbf{T}(x + \Delta x, t)$ and $-\mathbf{T}(x, t)$ (see Fig. 23.6). Moreover, according to Hooke's law, $|\mathbf{T}(x, t)|$ does not depend on x and t , i.e. $|\mathbf{T}(x, t)| = T_0$.

Defining T_1 as a relaxation time due to the action of the surrounded medium, we note that $\mathbf{F}_{\text{vis}}$ is directed parallel to the vertical coordinate axis and can be modelled as being proportional to $1/T_1 \partial u / \partial t$.

Let us denote by $f(x, t)$ the density of an external force $\mathbf{F}_{\text{ext}}$ acting on the membrane point x at time t and directing along the vertical axis. For a description of our electromagnetic candidate, see Section 23.3.5.

Thus, projecting Eq. (23.3) onto the vertical coordinate axis and taking the preceding relations into account, we get the equality

$$\begin{aligned} \rho \Delta x \frac{\partial^2 u}{\partial t^2} &= T(x + \Delta x, t) \sin(\alpha(x + \Delta x)) - T(x, t) \sin \alpha(x) \\ &\quad - \frac{2\rho}{T_1} \Delta x \frac{\partial u}{\partial t} + f(x, t) \Delta x. \end{aligned} \quad (23.4)$$

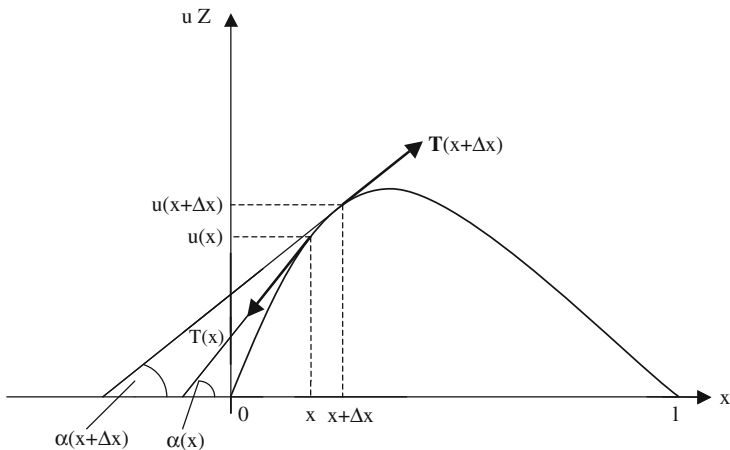


Fig. 23.6 Tension along the plasma membrane

In the context of our approximation

$$\sin(\alpha) = \frac{\tan(\alpha)}{\sqrt{1 + \tan^2(\alpha)}} \approx \tan(\alpha) = \frac{\partial u}{\partial x},$$

and, consequently, equality (23.4) takes the form

$$\frac{\partial^2 u}{\partial t^2} = \frac{\mathbf{T}_0}{\rho \Delta x} \left[\frac{\partial u(x + \Delta x, t)}{\partial x} - \frac{\partial u(x, t)}{\partial x} \right] - \frac{2}{T_1} \frac{\partial u}{\partial t} + \frac{1}{\rho} f(x, t). \quad (23.5)$$

Passing in (23.5) to the limit as $\Delta x \rightarrow 0$ we arrive at

$$\frac{\partial^2 u}{\partial t^2} + \frac{2}{T_1} \frac{\partial u}{\partial t} - c_s^2 \frac{\partial^2 u}{\partial x^2} = \frac{1}{\rho} f(x, t), \quad (23.6)$$

where $c_s^2 = \frac{\mathbf{T}_0}{\rho}$ is the speed of sound in the membrane.

23.3.3 The 2D Case

Similar to the 1D case we assume that in equilibrium state our plasma membrane lies in a subspace XY (see Fig. 23.7) and $u = u(x, y, t)$ denotes the membrane

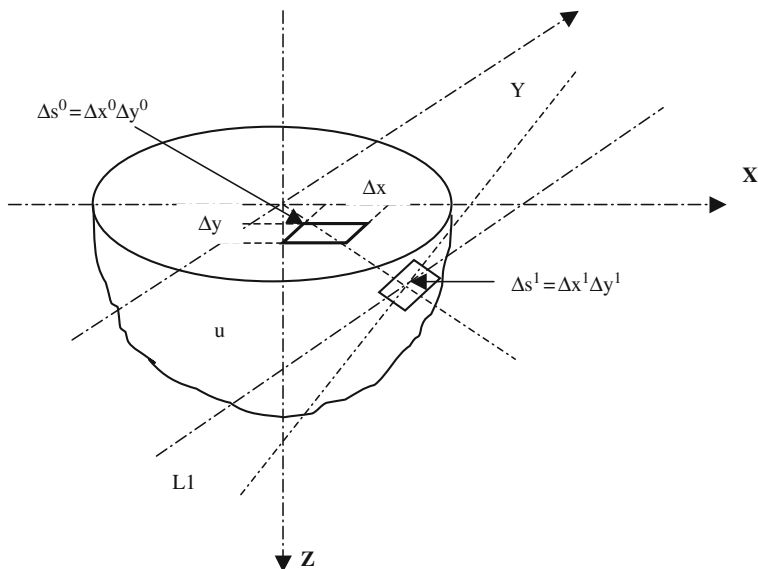


Fig. 23.7 Notations for membrane displacement from equilibrium state at the point (x, y) and at time t

displacement from equilibrium state at the point (x, y) and at time t . We will consider only small deformations such that

$$\left(\frac{\partial u}{\partial x}\right)^2 \ll 1, \quad \left(\frac{\partial u}{\partial y}\right)^2 \ll 1. \quad (23.7)$$

Let $d\sigma$ be the unit length of some closed path lying on the membrane surface, and let P be a point belonging to $d\sigma$. Then the unit $d\sigma$ is subjected to the tension force $\mathbf{T}d\sigma$, where $\mathbf{T} = \mathbf{T}(x, y, t)$ denotes the surface tension. Due to absence of the membrane resistance to bending and shear, we can say that the vector $\mathbf{T}$ always lies on the hyperplane L_1 tangential to the membrane surface at the point P , and $\mathbf{T}$ is orthogonal to $d\sigma$ (see Fig. 23.7).

In addition, inequalities (23.7) guarantee that the tangential hyperplane L_1 lies almost parallel to the hyperplane XY . To prove this statement, we have to show that the length of the projection of the vector $\mathbf{T}(x, y, t)$ onto XY is approximately equal to $|\mathbf{T}(x, y, t)|$. Indeed, by definition

$$|\text{Projection}_{XY} \mathbf{T}(x, y, t)| = |\mathbf{T}(x, y, t)| \cos(\beta),$$

where β stands for the angle between the tension vector $\mathbf{T}$ and hyperplane XY . It is easy to see that β is not bigger than the angle γ between the tangent hyperplane L_1

and XY . Therefore,

$$\cos(\beta) \geq \cos(\gamma) = \left[1 + \left(\frac{\partial u}{\partial x} \right)^2 + \left(\frac{\partial u}{\partial y} \right)^2 \right]^{-1/2} \approx 1,$$

and, consequently,

$$|\text{Projection}_{XY} \mathbf{T}(x, y, t)| \approx |\mathbf{T}(x, y, t)|.$$

Hooke's law guarantees that $|\mathbf{T}(x, y, t)|$ does not depend on the t -variable, whereas the orthogonality of $\mathbf{T}(x, y, t)$ and $d\sigma$ provides the independence of $|\mathbf{T}|$ on the variables x and y as well. It means that

$$|\mathbf{T}(x, y, t)| = \mathbf{T}_0 = \text{const.}$$

Now, considering a rectangle unit $ds = \Delta x \Delta y$ on the membrane surface, we can write the restoring force acting on this unit as

$$\begin{aligned} \mathbf{T}_0 \Delta y \left[\left(\frac{\partial u}{\partial x} \right) \Big|_{x+\frac{\Delta x}{2}} - \left(\frac{\partial u}{\partial x} \right) \Big|_{x-\frac{\Delta x}{2}} \right] + \mathbf{T}_0 \Delta x \left[\left(\frac{\partial u}{\partial y} \right) \Big|_{y+\frac{\Delta y}{2}} - \left(\frac{\partial u}{\partial y} \right) \Big|_{y-\frac{\Delta y}{2}} \right] \\ = \mathbf{T}_0 \Delta y \frac{\partial^2 u}{\partial x^2} \Delta x + \mathbf{T}_0 \Delta x \frac{\partial^2 u}{\partial y^2} \Delta y = \mathbf{T}_0 \left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} \right) \Delta x \Delta y. \end{aligned}$$

It remains to describe the external and viscosity forces acting on ds . Similar to the 1D case, $f(x, y, t)$ denotes the density of the external force $\mathbf{F}_{\text{ext}}$ at the point x and at time t . It is directed orthogonally to the membrane surface, while $\mathbf{F}_{\text{vis}}$, directed opposite to the vector $\mathbf{F}_{\text{ext}}$, is proportional to the velocity $\partial u / \partial t$.

Let $\rho(x, y)$ denote the membrane surface density, then the mass of the unit ds is equal to $\rho(x, y) \Delta x \Delta y$. Finally, defining a relaxation time due to the action of the surrounded medium by T_1 , we can write the variant of Eq. (23.3) for 2D membranes as follows:

$$\rho \Delta x \Delta y \frac{\partial^2 u}{\partial t^2} = \mathbf{T}_0 \left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} \right) - \frac{2\rho}{T_1} \Delta x \Delta y \frac{\partial u}{\partial t} + f(x, y, t) \Delta x \Delta y. \quad (23.8)$$

After elementary transformations, Eq. (23.8) takes the form

$$\frac{\partial^2 u}{\partial t^2} + \frac{2}{T_1} \frac{\partial u}{\partial t} - c_s^2 \left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} \right) = \frac{1}{\rho} f(x, y, t), \quad c_s^2 = \frac{\mathbf{T}_0}{\rho}. \quad (23.9)$$

23.3.4 Further Approximations

Having Eqs. (23.6) and (23.9) at hand, we observe that the process of dimple forming is quasi-static, i.e.

$$\frac{\partial^2 u}{\partial t^2} \ll 1.$$

The latter means that we can neglect this term in both equations.

It should also be pointed out that $u = |Du| = 0$ at those membrane points where there is no influence of the external force. Here Du denotes the spatial gradient of the displacement u .

Recall that the characteristic function χ_E of a set E is defined by

$$\chi_E(z) = \begin{cases} 1, & \text{for } z \in E, \\ 0, & \text{for } z \notin E. \end{cases}$$

Taking into account all the above remarks we get the following model equations for the dimple forming in the 1D- and 2D cases, respectively:

$$\frac{2}{T_1} \frac{\partial u}{\partial t} - c_s^2 \frac{\partial^2 u}{\partial x^2} = \frac{1}{\rho} f(x, t) \chi_{\{u>0\}}, \quad (23.10)$$

$$\frac{2}{T_1} \frac{\partial u}{\partial t} - c_s^2 \left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} \right) = \frac{1}{\rho} f(x, y, t) \chi_{\{u>0\}}. \quad (23.11)$$

23.3.5 Lorentz Force

Clearly, there are a variety of external forces resulting from the electromagnetic field wave. One can expect that they all play together in forming the dimple. However, taking our point of departure in an alternating current, we shall concentrate on the *Lorentz force* (implicitly deal with the *Coulomb force*), but discard the *dipole electric force*, the *magnetic force* and the *van der Waals force* for now.

23.3.5.1 Peculiarity of the Lorentz Force

In our physiological context, the peculiar role of the Lorentz force is that it exerts its action in one fixed direction, the direction of the propagation of the field wave, even when it is related to an alternating current. Roughly speaking, it is the sophistication and power of the electromagnetic aspects of the regulated exocytosis that a relatively weak and extremely low frequent electrodynamic wave can transport energy along a straight line over a large intracellular distance and exert its action on the charged phospholipid molecules in the plasma membrane. These charged molecules make

a substantial part of the eucaryotic plasma membrane, around 11% according to Rosenheck [19].

High-Voltage Devices: Quantitative Comparison of Electric Field Strengths in Electrical Power Plants and Animal Cells

A conventional 36.5 MW, 120 rotations per minute, 6,600 V High Tension Synchronous (HTS) generator yields a field strength of 100,000 V/m.

For comparison, in animal cells we consider the electrostatic field over the plasma membrane of 7 nm thickness and a potential of about 70 mV. That yields a field strength

$$|E| = \frac{70 \times 10^{-3} \text{V}}{7 \times 10^{-9} \text{m}} = 10^7 \frac{\text{V}}{\text{m}},$$

which is a 100 times stronger than the field strength generated in an electric high-power station.

Note that electric power engineering has to handle electromagnetic fields oscillating with low and very low frequencies. Therefore, in power engineering, the electromagnetism can be split into its electric and its magnetic character for optimizing the design of large machinery. Low frequent dynamic oscillations can yield (travelling) field waves, an aspect interesting for biophysics.

More to read: M. Koch, <http://www.feldkraft.de/>

Added by the editors

23.3.5.2 Energy Estimates

It may be instructive to have a rough idea of the scale of the electric forces and related energies around the dimple formation, see also the box. Based on the estimates given by Rosenheck l.c., we have a surplus of about 10^{10} (negative) charges per 1000 nm^2 plasma membrane area, i.e. about 10^9 charge carriers around the dimple top stretching over an area of approximately 100 nm^2 . The energy of one charged phospholipid molecule was calculated by Rosenheck l.c. as being $10^{-19} \text{Ws} = 10^{-19} \text{Nm}$. Dividing by a characteristic length of 10 nm we obtain a force of around 10^{-11}N , i.e. a total force of 10^{-2}N exerted on the dimple region.

For comparison, the gravitational force of the mass 10^{-21}kg of a dimple of about 10^3 nm volume (and specific weight comparable to water) would only sum up to $10^{-21} \times 9.8 \sim 10^{-20} \text{N}$. For another comparison, we refer to the Koch–Stetter electromagnetic field wave generator. For now, its B-field is of about 35 mT (milliTesla) and the exerted forces are visible and can be measured.

To sum up, for the making of the dimple we are investigating electric forces which surpass by large the above estimated 10^{-20}N .

23.3.5.3 Model Quantities

Our first model quantity is the capacitive reactance X_C as scalar function of place and time, depending on the shape of the dimple. Our second model quantity is the time-dependent (namely, dimple shape and X_C dependent) amplitude $\widehat{\mathbf{B}}$ of the magnetic flux density.

23.3.5.4 Model Equations

As mentioned before, we have two model levels:

- (I) There must be a variational equation, minimizing an energy functional or another related cost functional which gives the change and spatial distribution of X_C .
- (II) As discussed above, there are also elastic and viscous forces resisting the necessary re-packing of the lipid heads under deformation and pulling the plasma membrane back in the more smooth non-dimple form, interrupting exocytosis when the electrical and magnetic production is interrupted.

23.3.5.5 The Lorentz Force

Our favourite external force for making the dimple is the Lorentz force

$$\mathbf{F}_L = q\mathbf{E}_0 + q(\mathbf{v} \times \mathbf{B}) - \gamma\mathbf{v}, \quad (23.12)$$

where q is the charge (i.e. twice the number) of the released Ca^{2+} ions; $\mathbf{E}_0$ is the background electric field, here assumed to be zero; $\mathbf{B}$ is the magnetic flux density; $\mathbf{v}$ is the velocity of the charged dimple particles with respect to the coordinate system in which the quantities $\mathbf{F}$, $\mathbf{E}_0$ and $\mathbf{B}$ are calculated, i.e. $\mathbf{v}$ is up to the sign the wandering velocity of the directed electromagnetic field wave; and $\gamma\mathbf{v}$ is the friction force under propagation. Once again, Eq. (23.12) is written in SI. In the CGS system, common in physics, the term $q(\mathbf{v} \times \mathbf{B})$ has to be divided by c , the velocity of light in vacuum.

Note that we have for a (moving) field wave (see, e.g. [37, Chapter 13])

$$\mathbf{B}(\mathbf{x}, t) = \widehat{\mathbf{B}} \cos(\mathbf{k} \cdot \mathbf{x}(t) - k v(\mathbf{x}, t) t) + \mathbf{B}_{\text{DC}}, \quad (23.13)$$

$$\mathbf{E}(\mathbf{x}, t) = \mathbf{v}(\mathbf{x}, t) \times \mathbf{B}(\mathbf{x}, t). \quad (23.14)$$

Here, $\mathbf{B}_{\text{DC}}$ denotes the background B-field corresponding to the direct current $\mathbf{E}_0$, $\mathbf{x} = (x, y, z)$ denotes a position, $\mathbf{k}$ denotes the wave vector with $k = |\mathbf{k}|$ and $v(\mathbf{x}, t) = |\mathbf{v}(\mathbf{x}, t)|$.

The Lorentz force of Eq. (23.12) can be inserted into the general balance equation (23.3), into the 1D model equations (23.4), (23.6), simplified to (23.10), and into the 2D model equations (23.8), simplified to (23.11).

23.3.5.6 Work Equation

In Section 23.2.4, we have sketched the feedback mechanism of the dimple forming and explained why and how the forming of the dimple strengthens the electromagnetic field wave. It is beyond the range of this chapter to model that mechanism in detail as a free boundary problem. Basically, to relate our defining quantity X_C to the listed balance equations, we shall express the power in terms of X_C and then derive an integral for the electromagnetic energy density (per volume), where X_C enters. The details will be worked out separately.

23.4 Apposite Results on Parabolic Obstacle Problems

At the end of Section 23.3.4, we introduced the model equations (23.10) and (23.11) that can be treated as parabolic free boundary problems (FBP).

23.4.1 Review of Free Boundary Problems

The expression *free boundary problem* means that we deal with a problem with two a priori unknown objects: an unknown *set* coming up in a *solution* of a partial differential equation.

A typical example is the *Stefan problem* describing the melting of an ice cube in a glass of water. If ice begins to melt then the region occupied by water will grow and the interface – surface between the ice and the water (it is called the free boundary) – will move and change its shape, see, e.g. Friedman [38, Sect. 1.9]. As another typical example of FBP we mention the flame propagation problem describing the evolution of the flame front, see the various contributions in [39, Chapter 8].

Using a common transformation of the independent variables, we may normalize the coefficients and so reduce our model equations (23.10) and (23.11) to the following problem:

$$\begin{cases} \Delta u(x, t) - \frac{\partial u}{\partial t}(x, t) = f(x, t)\chi_{\{u>0\}}, \\ u(x, t) \geq 0 \end{cases} \quad \text{a.e. in } \mathcal{D}, \quad (23.15)$$

where a.e. means almost everywhere, (x, t) denotes the points in $\mathbb{R}^n \times \mathbb{R}$ with the space variable $x = (x_1, \dots, x_n)$ belonging to $\mathbb{R}^n$ and the time variable t belonging to $\mathbb{R}$, Δ is the Laplace operator defined as

$$\Delta u = \sum_{i=1}^n \frac{\partial^2 u}{\partial x_i^2},$$

$\chi_{\{u>0\}}$ is the characteristic function of the set $\{(x, t) \in \mathbb{R}^{n+1} \mid u > 0\}$ (see Section 23.3.4 for the precise definition), $\mathcal{D}$ is a given open set in $\mathbb{R}^{n+1}$ and u is a locally bounded weak (i.e. in the distributional sense) solution.

Observe that $\{u > 0\}$ is a priori an unknown open subset of $\mathcal{D}$. We denote by Γ the intersection of $\mathcal{D}$ with the *boundary* of the set $\{u = 0\}$. We will call Γ the *free boundary*. For our dimple forming, we have $\Gamma = \{\Gamma(t), \{t\}\}_{t \geq 0}$ of Fig. 23.3.

If, additionally, the condition

$$\frac{\partial u}{\partial t} \geq 0 \quad \text{a.e. in } \mathcal{D} \quad (23.16)$$

is satisfied, then our FBP (23.15) becomes the Stefan problem mentioned above. Inequality (23.16) means that our dimple is formed without “returning back”. In general, however, we cannot guarantee (23.16) from the assumptions given in Sections 23.3.2 and 23.3.3 only.

For the function $f(x, t)$, we assume that

- (1) f is *non-degenerate* in $\mathcal{D}$, i.e. there exists $\delta_0 > 0$ such that $f(x, t) > \delta_0$ for any $(x, t) \in \mathcal{D}$;
- (2) f is *Hölder continuous* in $\mathcal{D}$ with some $\alpha \in (0, 1)$, i.e. f is a bounded continuous function in $\mathcal{D}$, and for all points (x, t) and $(y, s) \in \mathcal{D}$ such that $(x, t) \neq (y, s)$ we have the inequality

$$\frac{|f(x, t) - f(y, s)|}{(|x - y|^2 + |t - s|)^{\alpha/2}} < \infty.$$

23.4.2 Qualitative Properties of Solutions

Let u be a solution of FBP (23.15), let $z^0 = (x^0, t^0) \in \mathcal{D}$, let $\rho > 0$ be sufficiently small and let $Q_\rho(z^0) := \{|x - x^0| < \rho\} \times (t^0 - \rho^2, t^0)$. The following estimates provide us with information about the behaviour of our displacement u near the interface between the sets $\{u > 0\}$ and $\{u = 0\}$, i.e. near the free boundary.

- There exists a constant $c = c(n) > 0$ such that

$$\sup_{Q_\rho(z^0)} u \geq c(n)\rho^2. \quad (23.17)$$

This nondegeneracy estimate holds true for all points z^0 belonging to the closure of the set $\{u > 0\}$ and for all ρ sufficiently small.

Moreover, our solution u has quadratic growth near the free boundary.

- There exists a constant $C > 0$ such that

$$\sup_{Q_\rho(z^0)} u \leq C\rho^2. \quad (23.18)$$

This nondegeneracy estimate holds true for all free boundary points $z^0 \in \Gamma$ and for all ρ sufficiently small.

- There exists a universal constant $M > 0$ such that

$$\sup_{Q_\rho(z^0) \cap \{u>0\}} \left(\left| \frac{\partial^2 u}{\partial x_i \partial x_j} \right| + \left| \frac{\partial u}{\partial t} \right| \right) \leq M \quad (23.19).$$

This inequality holds true for all free boundary points $z^0 \in \Gamma$ and for all ρ sufficiently small.

For $f(x, t) = \text{const}$ all these three statements were proved in [40]. The case of general $f = f(x, t)$ was considered in [41] for $n > 1$ and in [42] for $n = 1$.

The preceding estimates (23.17), (23.18), and (23.19) indicate that characteristic base diameter, growth rate, depth and time of the dimple forming are mathematically well defined and, therefore, these values should, in principle, be measurable in experiment, see Section 23.5.3.

23.4.3 Classification of Blow-Up Limits in $\mathbb{R}^{n+1}$

The idea is to use *blow-up* sequences, which are a kind of zooms, and to look at the “infinite zoom”. Suppose that u is a solution of the problem (23.15), $z^* = (x^*, t^*)$ is a free boundary point and $f(x^*, t^*) \neq 0$. For $\lambda > 0$ consider the functions

$$u_\lambda(x, t) := \frac{u\left(x^* + x \frac{\lambda}{\sqrt{f(x^*, t^*)}}, t^* + t \frac{\lambda^2}{f(x^*, t^*)}\right)}{\lambda^2}, \quad \text{for } (x, t) \in \mathcal{D}_\lambda := \frac{1}{\lambda} \mathcal{D}.$$

There exists a sub-sequence $\{\lambda_k\}$ converging to zero such that the blow-up sequence $\{u_{\lambda_k}\}$ converges to one of the following blow-up limits:

- $u_0 = u_{0,e}(x, t) := \frac{1}{2}(x^T \cdot \mathbf{e})^2$, for a unit vector $\mathbf{e}$, where $x^T \cdot \mathbf{e}$ denotes the scalar product in $\mathbb{R}^n$,
- $u_0 = u_{0,m}(x, t) = mt + x^T \cdot \mathcal{M} \cdot x$, where m is a constant and $\mathcal{M}$ is a $(n \times n)$ -matrix satisfying $\text{Tr} \mathcal{M} = m + 1$.

Observe that the blow-up limits can (in general) depend on the choice of the sub-sequence $\{\lambda_k\}$. But it should be emphasized that in view of the non-negativity of u , the limit function u_0 is the unique non-negative distributional solution of

$$\Delta u - \frac{\partial u}{\partial t} = \chi_{u>0} \quad \text{a.e. in } \mathbb{R}^n \times (-\infty, t^*).$$

This means that in the second case m and $\mathcal{M}$ are defined uniquely as well.

For $f(x, t) = \text{const}$ these results were obtained in [40]. For the general case $f = f(x, t)$ we refer the reader to [41].

23.4.4 Classification of the Free Boundary Points

Going to the regularity properties of the free boundary we observe that

- The free boundary is a closed set of zero $(n + 1)$ -Lebesgue measure.
- The free boundary $\Gamma = \{\text{“regular points”}\} \cup \{\text{“singular points”}\}$.
- The set of the singular free boundary points is closed.

The *singular points* are defined as the free boundary points for which there exists a blow-up limit of the second type, i.e. $u^0(x, t) = mt + x^T \cdot \mathcal{M} \cdot x$. The set $\Gamma \setminus \{\text{“singular points”}\}$ is the set of regular points. For singular points and for $k = 0, \dots, n$ the sets $S(k)$ are considered additionally, where $S(k)$ is defined as the set of singular points such that $\dim \text{Kern } \mathcal{M} = k$ and the smallest of the k non-zero eigenvalues is bounded from below by a fixed positive constant.

The complete classification of all free boundary points can be given via a relatively new approach introduced by Weiss [43]. For a solution u of FBP (23.15) and for a free boundary point $z^* = (x^*, t^*)$ consider the following energy functional:

$$W(\tau, z^*, u, f) := \frac{1}{\tau^4} \int_{t^* - 4\tau^2}^{t^* - \tau^2} \int_{|x - x^*| < \tau} \left(|\nabla u|^2 + 2fu + \frac{u^2}{t - t^*} \right) G(x - x^*, t^* - t) dx dt,$$

where $G(x, t)$ is the heat kernel.

The functional W has the following remarkable properties:

- $\lim_{\tau \rightarrow 0^+} W(\tau, z^*, u, f)$ exists and is finite;
- There are only two possible values for $\lim_{\tau \rightarrow 0^+} W(\tau, z^*, u, f)$, namely

$$\lim_{\tau \rightarrow 0^+} W(\tau, z^*, u, f) = \begin{cases} A_n, & \text{if } z^* \text{ is a regular point} \\ 2A_n, & \text{if } z^* \text{ is a singular point} \end{cases}$$

for some constant $A_n > 0$ depending only on the dimension n .

Finally, we observe that

- Around regular points the free boundary is a smooth graph.
- Singular points belonging to $S(n)$ are isolated.
- $S(n)$ is contained locally in a C_x^2 -graph in space.
- $S(k)$ for $0 \leq k \leq n - 1$ is contained locally in a k -manifold of class $C_{x,t}^{1/2}$.

For all results concerning the regular points we refer to [40]. The results about singular points were proved in [41] (see also [44]).

Experiment and Discovery

In science, we meet various ways of relating experiments and discovery and very different perceptions of that process. Physics has (naturally had to) developed what other scientists consider a rather crude iteration cycle between *theory*, *model* and *experiment*: first we mess around making some observations; then we specify and embed the observation into a mathematical model; after this we create a theory by relating our observations and our model to other observations and other models; all that gives rise to new questions and new experiments, partly model-based, partly again messing around – and the cycle begins again.

A related view, shared in systems biology, upon experiments is to distinguish between *exploratory* experiments, anteceding models and theory, and *critical* experiments confirming or falsifying a model or a theory. For use in medicine and biology, the immunologist and 1960 Nobel Laureate Peter Medawar elaborates on that distinction and discerns four kinds of experiments.

A first kind of experimentation is one in the original *Baconian* sense, i.e. any organized experience or happening, devised with ingenuity or skill. It is the consequence of “trying things out” or even of merely messing around. Baconian experiments try to answer the question “I wonder what would happen if ...”. Such experimentation proved productive from the time of alchemy until the discovery of high-temperature superconducting a few decades ago.

A second kind of experimentation is what Medawar baptized *Aristotelian*: these experiments are also contrived, but not out of curiosity but for supporting an existing model or theory or just to demonstrate its basic essence by an observatory example, like Galileo’s observation of the Jupiter moons in confirmation of the Copernican planetary system and NASA’s recent Lunar Laser Ranging Test of the invariance of c .

The ideal of modern science is a third kind of experimentation, the *Galilean* or *critical experiment*. The main goal is to provide striking evidence against a previous erroneous perception. The aim is clarification by falsification, like Galileo’s balls rolling down an inclined plane in an almost frictionless environment to refute mass dependence of the velocity of falling bodies (and thereby establishing constant acceleration) or the famous Michelson–Morley experiment to reject the theory of a luminiferous aether.

Finally, there are the *Kantian* experiments – the thought experiments: “Let’s see what would follow if we took a somewhat different view...”. Kantian experimentation requires no apparatus except sometimes a computer. From discussions between Bohr and Einstein, series of Kantian experiments are documented. Einstein himself attributed his discovery of Special Relativity to shared fantasy with his first wife of sitting together on the tip of a light beam.

Further Reading:

Medawar, PB (1979) Advice to a young scientist, Basic Books Inc., New York, NY

Added by the editors

23.5 Conclusions

Our conclusions consist of preliminary findings which will require further experiments and measurements to be confirmed – or falsified.

23.5.1 *Summary of (Partly Speculative) Working Hypotheses*

For better reading, we begin this closing section by summarizing our assumptions and choices of emphasis:

1. Among all relevant aspects of the well- and malfunctioning of pancreatic β -cells, we focus on a single membrane process, the lipid bilayer fusion event.
2. We suppose that future imaging will prove the making of a plasma membrane dimple before the making of the fusion pore.
3. We assume that the dimple making is essential for the performance of regulated exocytosis also in β -cells.
4. We suspect that maintaining the fusion pore and continuing release of the content of the insulin granules will be interrupted when the dimple is not preserved.
5. We argue for a long-distance regulation of the dimple making (and, hence, the secretion process), i.e. we claim that the docking of readily releasable insulin granules at the plasma membrane and the consecutive making of the fusion pore and the release of the hormone molecules are induced not as a purely local phenomenon and hence cannot be explained solely by glucose stimuli and corresponding Ca^{2+} influx through ion channels in the neighbourhood of the release site. Instead of that, we claim that stimuli and ion influx also far from the exocytosis site and mediated by intra-cellular signalling and energy transport will be decisive for initiating and maintaining regulated exocytosis even at one isolated site.
6. We point to a low frequent electromagnetic field wave as a possible regulator of exocytosis.
7. We suppose that the electromagnetic field will be closed over the plasma membrane due to the iron content of enzymes embedded between the phospholipids of the plasma membrane.
8. We assume that the electrical activity of the mitochondria is equally important as the electrical activity at the plasma membrane. We suppose that the mitochondria sequester and release Ca^{2+} ions in a self-regulated way which builds an electromagnetic field and generates a directed (travelling) field wave.
9. We suppose that the synchronization of the electrical activity of neighbouring mitochondria is due to energy efficiency.
10. While many aspects of β -cell function will require an analysis of their collective functioning in the Langerhans islets, we conjecture that essential aspects of regulated exocytosis can be observed on the level of a single cell.

23.5.2 *The Findings*

We have provided a mathematical model for the initiation of regulated exocytosis and the making of the fusion pore. The model relates the geometry and the dynamics of one single membrane process, namely the forming of an inward oriented dimple in the plasma membrane before the fusion event, with electromagnetic features of intracellular calcium oscillations. The model suggests a new explanation for the observed flickering of regulated exocytosis, the vanishing of the first phase of secretion in stressed or tired β -cells and the final halt of all secretion in overworked dysfunctional cells: the electromagnetic free boundary model points to the lack of stability and coordination of the intracellular Ca^{2+} oscillations prior to the bilayer membrane vesicle fusion. We recall that the field character of these oscillations is magnetic (therefore transferring energy to the fusion site at the plasma membrane without any loss). It must be distinguished from the widely studied Ca^{2+} influx changing the electrostatic potential across the plasma membrane and accompanying regulated exocytosis.

The model is based solely on physical First Principles. All parameters have a biophysical meaning and can, in principle, be measured.

23.5.3 *Suggested Experiments and Measurements*

At the present stage of our knowledge, the mathematical and biophysical *correctness* of our model does not prove its *relevance* for explaining the phenomena it claims to explain. To decide whether the here described phenomena and effects are *dispensable* or *decisive* for regulated exocytosis, the scales of the ion oscillations, the electromagnetic fields, the acting forces, the entering material constants and the characteristic times and lengths must be determined.

Hence, the framework of our electromagnetic free boundary problem for the dimple making suggests the following array of experiments and measurements:

1. We shall observe the Ca^{2+} oscillations prior to the bilayer membrane-vesicle fusion also in pancreatic β -cells and determine their spatial and temporal character. In particular, the observations must
 - check the intracellular origin of the oscillations; we may, e.g. deliberately silence (empty) some types of organelles by adding suitable agents, see, e.g. Fridlyand et al. [45];
 - identify the participating organelles (Ca^{2+} storages) and
 - decide about the orientation (the direction) of oscillations; the frequency, depending on stimulus; and the distinction between almost simultaneous oscillations pointing in different directions as their selected sites for the making of the fusion pore.
2. We shall modulate the oscillations by submitting the cells to an external field generator with variable frequencies, to prove the magnetic character of the field wave associated with the oscillations.

3. We shall determine the surface tension in the plasma membrane of living cells. In particular, we shall measure and/or calculate the bending rigidity and stretching elasticity under “repacking” of the ball-shaped heads of the inner lipids under area changes. For living cells, we expect that these magnitudes are substantially larger than for model membranes, e.g. due to osmotic pressure in living cells, see Henriksen and Ipsen [46].
4. We shall measure the cytosol viscosity close to the plasma membrane, i.e. update the classic study [47] by Bicknese et al. and locate actin filaments blocking for unwanted docking of the insulin granules at the plasma membrane.
5. We shall check Rosenheck’s estimate (l.c.) for charged molecules in the plasma membrane.
6. We shall estimate the content of (para-, not necessarily ferro-)magnetizable Fe atoms and crystals in the membranes to determine their magnetic momentum.
7. We shall estimate the distribution of the inhomogeneities of the magnetic field near the plasma membrane.
8. We need precise electron or atomic force microscope slices of the dimple making and of the degree of its singularity in β -cells.
9. We shall measure by patch clamp technique the expected decrease of the capacitive reactance X_C under dimple forming.
10. We shall correlate the Ca^{2+} oscillations with the fusion events; in particular, we shall confirm the spatial and temporal coincidence of flickering of exocytosis with breakdowns of the field wave.
11. We shall demonstrate the absence or weakness of the Ca^{2+} oscillations after stimulation in stressed or tired β -cells.
12. After obtaining reliable values of all data involved in our mathematical model, we shall create a computer simulation of the free boundary problem to get a solution graphically (in the form of a surface). After that, we can compare the dimple images and the modelled surface.

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