Reviews of

126 Physiology Biochemistry and Pharmacology

With 32 Figures and 15 Tables

Springer-Verlag
Berlin Heidelberg New York London Paris
Tokyo Hong Kong Barcelona Budapest

ISBN 3-540-58477-3 Springer-Verlag Berlin Heidelberg New York

Library of Congress-Catalog-Card Number 74-3674

Typesetting: Macmillan India Ltd., Bangalore-25

SPIN: 10128258 27/3130/SPS – 5 4 3 2 1 0 – Printed on acid-free paper

Contents

Contractile Properties of Skeletal Muscle Fibers
in Relation to Myofibrillar Protein Isoforms
By R.L. MOSS, G.M. DIFFEE, and M. L. GREASER,
Madison, Wisconsin, USA
With 15 Figures 1

Hexokinases
By J.E. WILSON, East Lansing, Michigan, USA
With 10 Figures and 2 Tables 65

Molecular Chaperones
and Intracellular Protein Translocation
By J. RASSOW and N. PFANNER, Freiburg, Germany
With 6 Figures and 3 Tables 199

Heteroreceptor-Mediated Modulation of Noradrenaline
and Acetylcholine Release from Peripheral Nerves
By H. FUDER and E. MUSCHOLL, Mainz, Germany
With 1 Figure and 10 Tables 265

Subject Index 413

Indexed in Current Contents

Rev. Physiol. Biochem. Pharmacol., Vol. 126

Contractile Properties of Skeletal Muscle Fibers in Relation to Myofibrillar Protein Isoforms

R.L. Moss[1], G.M. Diffee[1], and M.L. Greaser[2]

Contents

1 Introduction 2

2 Physiological Assessment of Muscle Fiber Performance 4
- 2.1 Isometric Tension 5
- 2.2 Stiffness 10
- 2.3 Shortening Velocity 11
- 2.3.1 Determinants of Shortening Velocity 11
- 2.3.2 Effects of $[Ca^{2+}]$ on Shortening Velocity 13
- 2.3.3 Power Versus Load Curves 16
- 2.4 Tension Transients 18
- 2.4.1 Transients in Response to Rapid Changes in Length 18
- 2.4.2 Transients Following Photolysis of Caged Compounds 20
- 2.4.3 Tension Redevelopment Following Release and Restretch 21

3 Mechanical Effects Due to Natural Transitions in Isoform Content in Mammalian and Avian Muscles 24
- 3.1 Myosin Heavy Chains 24
- 3.1.1 Subunit Structure of Myosin 24
- 3.1.2 Variation of Twitch Kinetics with Myosin Heavy Chain Content 25
- 3.1.3 Variation of Shortening Velocity with Myosin Heavy Chain Content 26
- 3.2 Alkali Light Chains 30
- 3.3 Thin Filament Regulatory Proteins 32
- 3.3.1 Cooperative Activation of Tension 32
- 3.3.2 Effects of Isoforms of Troponin-T 34
- 3.3.3 Effects of Fast and Slow Isoforms of Troponin-C 36
- 3.4 Titin 37
- 3.5 Parvalbumin 38

4 Mechanical Effects Due to Experimental Manipulation of Isoform Content 41
- 4.1 Myosin Heavy Chains 41
- 4.1.1 Effects of In Vivo Stimulation on MHC Composition and Mechanical Properties of Muscle 42

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4.1.2 Effects of Altered Thyroid Status on MHC and Mechanical Properties 43
4.1.3 Effects of Activity Patterns on MHC and Mechanical Properties 43
4.2 Myosin Light Chain-2 45
4.3 Alkali Light Chains 46
4.4 Troponin-C 48
5 Relative Effects of Fast and Slow Isoforms of MHC on Shortening Velocity 49
6 Conclusions 52
References 53

1 Introduction

Contraction of vertebrate striated muscles results from cyclic interactions between the proteins myosin and actin, which in intact muscles are formed into parallel arrays of overlapping thick and thin filaments (Fig. 1). In resting muscles myosin cross-bridges from the thick filament interact with actin weakly or not at all due to inhibitory effects of the regulatory proteins troponin (Tn) and tropomyosin (Tm) bound to the thin filament. Stimulation of living muscle either by nerve activity or with electric current results in an increase in myoplasmic Ca^{2+} concentration and subsequent activation of thin filaments due to binding of Ca^{2+} to Tn (Fig. 2). The detailed mechanisms of activation are not known, but movement of Tm is almost certainly required for cross-

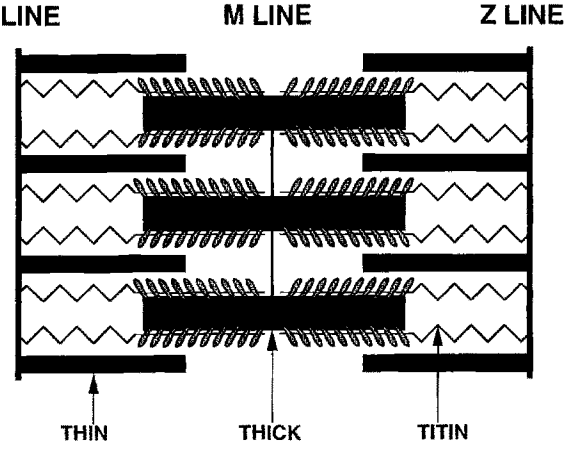

Fig. 1. Schematic diagram showing the spatial relationships of thick and thin filaments, titin, and Z-lines. Thick filaments are comprised primarily of myosin and a much smaller component of C-protein. Thin filaments are comprised primarily of actin, Tm and Tn. Detailed structure of thick and thin filaments is shown in Fig. 2

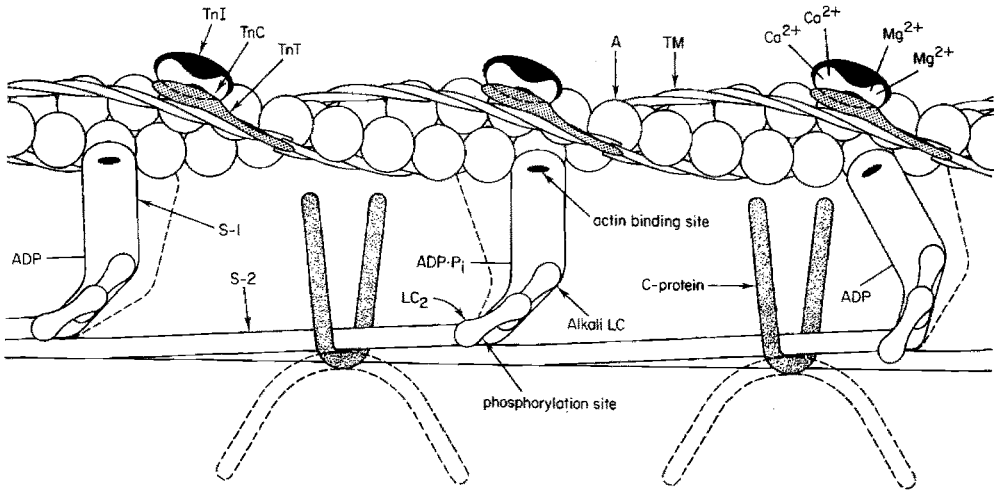

Fig. 2. Schematic diagram of thick and thin filaments drawn approximately to scale. A, Actin; Tm, tropomyosin; Tn, troponin; LC, light chain (LC_2 is also called the 20 kDa LC, the P-light chain, regulatory LC, or DTNB LC; the alkali LCs are also called "essential" LCs). C-protein is drawn in two different configurations since its position in the thick filament is not known (see Solaro 1986)

bridge interaction to proceed. While involvement of Ca^{2+} in regulating contraction is widely accepted, several additional factors contribute to the contractile properties of muscle. For example, within muscles of a given type, either fast-twitch or slow-twitch, the rate of tension development appears to be regulated primarily by the proportion of cross-bridges that are strongly bound, i.e., the rate of interaction becomes faster as the number of strongly bound cross-bridges increases (Swartz and Moss 1992). Physical factors, such as myoplasmic pH or the accumulation of inorganic phosphate (P_i), affect both the extent and rate of cross-bridge interaction.

Variable expression of protein isoforms among muscles of different types is a major determinant of contractile properties. Altered expression of myosin isoforms during development and by experimental intervention has been associated with differences in the rate of force development and the maximum velocity of shortening (Close 1964). Fast and slow isoforms of regulatory proteins appear to confer different sensitivities of contraction to Ca^{2+}. Finally, different isoforms of contractile and regulatory proteins are associated with differing degrees of responsiveness of muscle fibers to physical factors, such as reduced pH and elevated P_i, and probably accounts for much of the observed variation in susceptibility to fatigue among fiber types.

Skeletal muscles fibers have typically been categorized as fast or slow on the basis of contractile speed, acid or alkaline sensitivity of myosin ATPase activity, myosin isoform composition (as determined by gel electrophoresis or by immunoreactivity to antibodies specific for MHC isoforms), mitochondrial and glycolytic enzyme activity, or some combination of these methods (for review, see Pette and Staron 1990). While it is certainly possible to generally classify fibers as either slow or fast based on contractile speed, and fibers correspondingly have appropriate distributions of myosin isoforms (Gauthier and Lowey 1979), there are numerous cases in which various myosin isoforms are multiply expressed within a given single fiber (e.g., Mabuchi et al. 1984; Reiser et al. 1985a; Staron and Pette 1986). Based on results such as these it is now generally thought that muscle fibers form a continuum with respect to contractile speed and expression of myosin heavy chains (MHCs), and that fibers containing purely one type of myosin isoform are simply part of this continuum.

The primary purpose of this paper is to review the roles of protein isoform expression in modulating the mechanical properties of skeletal muscle and, where possible, to discuss the specific mechanisms of modulation. To limit the scope of the review we use only a few specific examples of modulation rather than attempting to describe all previous work in the field. For background to this review, there are several earlier reviews that provide excellent discussions of variations in myofibrillar protein expression in skeletal muscles during development and due to experimental interventions such as training, altered hormone status, and the imposition of stress upon limb muscles (Andreadis et al. 1987; Bandman 1992; Hoh 1991; Pette and Staron 1990; Pette and Vrbová 1992; Syrovy 1987; Wade and Kedes 1989).

2 Physiological Assessment of Muscle Fiber Performance

Assessment of contractile function in living skeletal muscle is based upon a variety of mechanical measurements which yield information about the regulation of cross-bridge interaction with actin, the overall cycling rate of cross-bridge interaction, and the kinetics of specific transitions between biochemical states in the interaction cycle (Fig. 3). Even relatively simple mechanical measurements such as tension and shortening velocity provide important information about the distributions of cross-bridges among weakly and strongly bound states and the kinetics of transitions between states. To facilitate discussion of mechanical effects

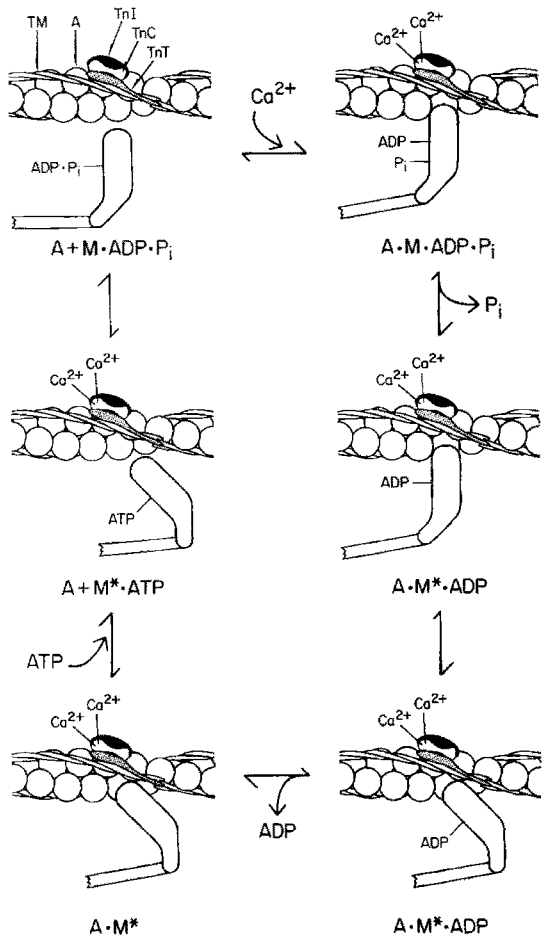

Fig. 3. Cross-bridge interaction cycle. A, Actin; M, myosin; P_i, inorganic phosphate; M*, myosin that has undergone the transition to the force generating state

due to changes in protein isoform content of muscles, this section discusses molecular mechanisms of mechanical behavior and the kinds of information that mechanical measurements provide. Other than tension and shortening velocity, remarkably few of these approaches have been used to probe the functional significance of variations in myofibrillar protein isoforms.

2.1 Isometric Tension

Tension developed by a muscle is directly related to the number of cross-bridges in strongly bound, tension-generating states. Biochemically, these states appear to be those in the cross-bridge cycle closely associated with or subsequent to the dissociation of inorganic phosphate (P_i) from the

actin/myosin-ADP-P_i complex (Fig. 3), as P_i release has been shown to be kinetically coupled to tension generation in skinned muscle fibers (Millar and Homsher 1990). Since the interaction of myosin with actin in muscle is regulated by Ca^{2+}, the number of cross-bridges bound to actin, and consequently tension, varies with the concentration of Ca^{2+} bathing the myofilaments. In living muscle a single, suprathreshold stimulus transiently increases myoplasmic Ca^{2+} to levels that are probably sufficient to saturate Ca^{2+} binding sites on the thin filament; however, at physiological temperatures, the Ca^{2+} transient is too brief to allow cross-bridges to achieve a steady-state distribution between force-generating and non-force-generating states, so that peak twitch tension is submaximal. During sustained, tetanic stimulation, myoplasmic Ca^{2+} is held at high levels, and tension ultimately reaches a maximum (Blinks et al. 1978). In skinned muscle preparations the membrane is permeabilized or removed physically to directly expose the myofilaments to the bathing solution. Activation of contraction requires addition of Ca^{2+} to a bathing medium that already contains Mg-ATP, a Ca^{2+} buffer such as EGTA, a pH buffer, and physiological salts. The steady isometric tension developed by skinned muscle preparations represents a steady-state distribution of cross-bridges between strongly and weakly bound states, and the number of strongly bound bridges can be varied by adding differing amounts of Ca^{2+} to this solution.

A number of physical factors can also affect the distribution of cross-bridges between weakly and strongly bound states, and these effects may differ between fiber types. For example, decreased pH reduces maximum Ca^{2+} activated tension in skinned fibers, but this effect is greater in fast-twitch than in slow-twitch fibers (discussed below). Other factors, such as alterations in $[P_i]$ or [ADP] also affect tension development, presumably by direct or indirect effects on the equilibrium constant for the P_i release step in the cross-bridge interaction cycle.

The primary mechanisms of altered force production by muscle fibers in vivo are changes in stimulus frequency or muscle length, or the onset of fatigue. (a) *Stimulus frequency*. Measurements of Ca^{2+} transients during a twitch suggest that sufficient Ca^{2+} is released to saturate TnC, the Ca^{2+} binding subunit of Tn (Blinks et al. 1978). But despite saturation of Ca^{2+} binding sites, twitch force is usually not the maximum a muscle can produce. The most likely explanation for this phenomenon is that the rate of interaction of cross-bridges with actin is too slow to establish a steady-state population of force generating bridges. However, with high-frequency tetanic stimulation $[Ca^{2+}]$ can be maintained at saturating levels for prolonged periods, and steady maximum tension is

achieved. The intervals between successive nerve impulses in vivo are ordinarily too long to yield steady tetanic tension but is sufficiently high to cause some summation of Ca^{2+} transients and tension in response to successive stimuli. (b) *Muscle length.* Under experimental conditions in which single muscle fibers are stimulated tetanically, isometric tension varies as a function of sarcomere length due to alterations in the amount of overlap of thick and thin filaments and consequent changes in the amount of cross-bridge interaction with actin. Tension falls dramatically at very short lengths due to structural interference with contraction, but these lengths are not reached under physiological conditions. The typical range of movement in vivo is only about 0.5 μm per sarcomere, which could account for reductions in tetanic force of approximately 20% below the maximum. In contrast, twitch tension shows a much different variation with sarcomere length (Close 1972a) than would be predicted on the basis of changes in filament overlap (Gordon et al. 1966). This result suggests that twitch tension is greatly influenced by factors other than overlap. One possibility is that twitch tension falls due to a reduction in sensitivity of myofilaments to Ca^{2+} at short sarcomere lengths. Consistent with this idea, tensions measured in skinned fibers at low $[Ca^{2+}]$ are reduced to much greater proportions at short lengths than tensions at high $[Ca^{2+}]$. (c) *Fatigue.* Fast-twitch muscle fibers, and to a lesser extent slow-twitch muscle, can be fatigued by short periods of intense work or prolonged periods of moderate work. Fatigue is generally defined as a reduction in maximum tension generating capability which is characterized by a reduced ability to perform work. The basis for fatigue varies (reviewed by Fitts 1994): fatigue in response to bursts of intense work appears to be due to effects of metabolites such as P_i and H^+ on the interaction of contractile proteins, while fatigue from long duration work may also involve failure of excitation-contraction coupling, perhaps at the junctions between t-tubules and sarcoplasmic reticulum. While [ATP] may be reduced during fatiguing stimulation, and [ADP] may be elevated, the magnitudes of these changes are not sufficient to alter the tension-generating capabilities of skeletal muscles (see Fitts 1994).

In considering mechanisms of altered force production in vivo it is important to recognize that activation of the thin filament not only involves Ca^{2+} binding to regulatory proteins (reviewed by Ashley et al. 1991) but also depends on the number of cross-bridges bound to actin (see Sect. 3.3.3), i.e., strongly bound cross-bridges increase both the rate of interaction of myosin with actin and the apparent binding affinity of TnC for Ca^{2+}. Thus, in the cross-bridge interaction scheme in Fig. 3,

conditions that increase the number of strongly bound cross-bridges further increase tension by cooperatively increasing the level of activation of the thin filament. The mechanism underlying this effect seems to be that force-generating cross-bridges increase the amount of Ca^{2+} bound to TnC. Alternatively, strongly bound cross-bridges may allow additional cross-bridges to bind, due perhaps to a direct effect on Tm. Cross-bridge sensitization of the thin filaments to Ca^{2+} has been shown in experiments in which a strongly binding cross-bridge analog (i.e., *N*-ethyl-maleimide Sl) that does not bear tension or hydrolyze ATP increased the Ca^{2+} sensitivity of tension in skinned fibers (Swartz and Moss 1992), as shown in Fig. 4.

Several physical factors appear to alter the distribution among weakly and strongly bound cross-bridge states and therefore alter twitch tension as a result of decreased Ca^{2+} sensitivity of isometric tension. These factors are discussed here in the context of experiments on skinned fibers in which free [Ca^{2+}] is directly controlled, since many of these interventions may also affect the amount of Ca^{2+} released by the sarcoplasmic reticulum during excitation-contraction coupling in living muscle.

Temperature. Increases in temperature increase the maximum tension that muscle fibers develop in solutions containing saturating levels of Ca^{2+}. At submaximal Ca^{2+} the observed effects of temperature on tension have not been consistent among all investigators, and the reasons for these differences are not yet known. For example, Stephenson and Williams (1981) found that increases in temperature from 5° to 25°C

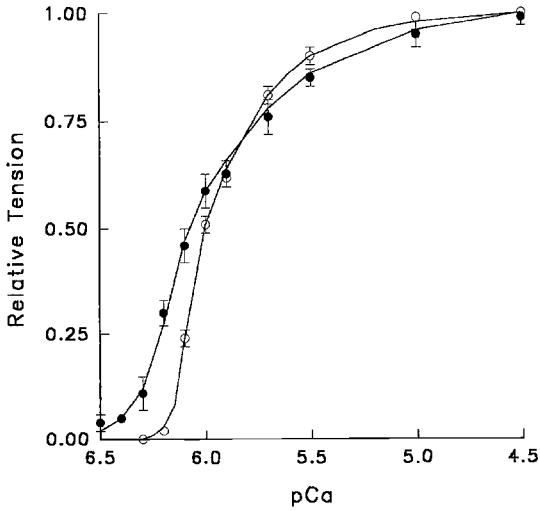

Fig. 4. Tension-Ca relationships in the presence and absence of NEM-S1, a strong-binding derivative of myosin S1. Tension-pCa relationships were determined in untreated skinned fibers from rabbit psoas muscles (open circles) and in the same fibers following treatment with 4 μ NEM-S1 (filled circles). Treatment with NEM-S1 increased the Ca^{2+}-sensitivity of tension and reduced the slope of the relationship. (Unpublished data from Zu, Swartz, Walker, and Moss)

(but not higher temperatures) reduced the Ca^{2+} sensitivity of tension in rat fast-twitch muscle fibers, i.e., tension at any given [Ca^{2+}] decreased when temperature was increased. Similarly, Ca^{2+} sensitivity of tension decreased in frog skinned muscle fibers when temperature was increased between 4° and 22°C (Godt and Lindley 1982). On the other hand, Sweitzer and Moss (1990) found that Ca^{2+} sensitivity of tension in rabbit skinned psoas fibers increased when temperature was raised from 10° to 20°C, which they attributed to a cooperative activation of the thin filament due to increased numbers of strongly bound cross-bridges. Increased temperature also appears to increase the force per attached cross-bridge, which increases total developed force and may itself increase the Ca^{2+} sensitivity of tension (Sweitzer and Moss 1990).

P_i and H^+. Both P_i and H^+ are products of ATP hydrolysis that accumulate during fatiguing stimulation (Fitts 1994), and each has been shown to reduce maximum isometric tension in skinned fibers (e.g., Metzger and Moss 1987). The mechanisms of these effects are not entirely clear, but at least in the case of elevated P_i they may involve alteration in equilibrium of the P_i release step, which is associated with force development. Whatever the specific mechanism, the decrease in maximum tension accompanying increases in either [P_i] or [H^+] is associated with reductions in the Ca^{2+} sensitivity of tension in both fast- and-slow-twitch fibers.

H^+ ion has at least two additional effects (Metzger and Moss 1990a) in skinned fibers that contribute to its effects in vivo. First, increased [H^+] actually reduces the force produced by each attached cross-bridge, an effect which is similar in fast- and slow-twitch fibers. Secondly, the effect of H^+ on maximum force is substantially greater in fast-twitch fibers, which may account for the lesser fatigue resistance of these fibers than in slow-twitch fibers.

Phosphorylation of Myosin LC_2. Tetanic stimulation of skeletal muscle results in increased peak tension during subsequent twitches, i.e., post-tetanic potentiation (PTP) of twitch tension. PTP is associated with phosphorylation of myosin LC_2 by a Ca^{2+}-calmodulin dependent myosin light chain (MLC) kinase (reviewed by Sweeney et al. 1993). While phosphorylation is a probable cause of increased twitch tension, it has no effect on maximum isometric tension in skinned fibers and modestly increases the Ca^{2+} sensitivity of tension. Increased twitch tension probably results primarily from an increased rate of cross-bridge cycling subsequent to light-chain phosphorylation (Metzger et al. 1989; Sweeney

and Stull 1990; Sweeney et al. 1993). Thus, in a given Ca^{2+} transient there is greater force generated by a muscle fiber having faster transitions to strongly bound cross-bridge states.

Involvement of Thick Filament Accessory Proteins. There is increasing evidence that C-protein (Hofmann et al. 1991a,b) and myosin LC_2 (Hofmann et al. 1990), proteins associated with the thick filament, influence the Ca^{2+} sensitivity of tension. Partial extraction of either protein increases the Ca^{2+} sensitivity of tension in skinned fibers. This result has been explained in terms of possible effects of each of these proteins to influence the flexibility of the myosin molecule, thereby regulating the availability of cross-bridges to actin and the number of cross-bridges that are strongly bound. However, it is not yet known whether these proteins have roles in determining the rate or amount of tension developed in vivo.

2.2 *Stiffness*

Conditions that change the tension-generating capabilities of a muscle may do so by changing the number of strongly bound, force-generating cross-bridges and/or by changing the force developed by each cross-bridge bound to actin. These possibilities can usually be distinguished by simultaneously measuring force and stiffness, since stiffness is thought to be a direct measure of the number of strongly bound cross-bridges (Ford et al. 1977). Stiffness ($\Delta F/\Delta L$) is assessed by measuring the change in force (ΔF) in response to a rapid sinusoidal or step change in length (ΔL). Since the working stroke of a cross-bridge during a single interaction with actin may be 0.5% or less of the length of a half-sarcomere (Ford et al. 1977), the length changes applied during stiffness measurements are generally 0.1% or less to minimize disruption of formed cross-bridges. Nevertheless, changes in overall muscle length change the strain of individual cross-bridges, which subsequently undergo transitions that result in recovery of force. Thus, length changes must generally be rapid (<1 ms duration) in order to avoid underestimating stiffness due to force recovery during the length change. The effects of recovery processes on stiffness are clearly evident in measurements of stiffness as a function of frequency of length change. At low frequencies measured stiffness is low because the force-generating transitions in the cross-bridge interaction cycle are fast relative to the length change, i.e., the muscle accommodates the length change by maintaining a relatively constant force.

As frequency is increased, stiffness increases because the rate of length change is progressively faster than the force generating transitions. Finally, at sufficiently high frequencies, the length change is so rapid that the cross-bridge head is virtually immobile during the length change. At these frequencies all of the length change is taken up by the elastic portions of the cross-bridge, and stiffness under these conditions directly assesses the numbers of cross-bridges strongly bound to actin. Since the cross-bridge is now an elastic structure, changes in force are coincident with changes in length, i.e., the signals are in phase.

The proportion of strongly bound cross-bridges under a variety of experimental conditions can be determined by measuring in-phase stiffness and comparing this value to in-phase stiffness measured while the muscle is in rigor, and 100% of cross-bridges are strongly bound. The interpretation of stiffness data is not necessarily simple even when force and length are in phase. For example, if an experimental condition or a change in protein isoform expression leads to a reduction in force but no change in stiffness, the straightforward conclusion is that the amount of force per attached cross-bridge is reduced. However, from these data it is not possible to determine whether the mean strain of force-generating cross-bridges is reduced, whether there has been a redistribution of cross-bridges between strongly bound force-generating states and strongly bound pre-force-generating states.

In the series of events that result in force development it appears likely that cross-bridges are first weakly bound to actin, then strongly bound, and finally strongly bound and force generating (Dantzig et al. 1988). Weakly bound cross-bridges rapidly detach from actin when length is changed (Brenner et al. 1982). Thus, if the frequency of stiffness measurements is kept to a few kilohertz, it is likely that only strongly bound cross-bridges contribute to stiffness; however, if the frequency is increased substantially (to approximately 10 kHZ) so that the rate of length change is fast relative to detachment of weakly bound cross-bridges, both weakly and strongly bound cross-bridges contribute to stiffness. By assessing stiffness at intermediate and very high frequencies it should therefore be possible to determine the distribution of non-strongly bound cross-bridges between weakly bound and unbound states.

2.3 Shortening Velocity

2.3.1 Determinants of Shortening Velocity

When the load on a muscle is less than its isometric tension generating capability, the muscle shortens and lifts the load at a velocity that varies

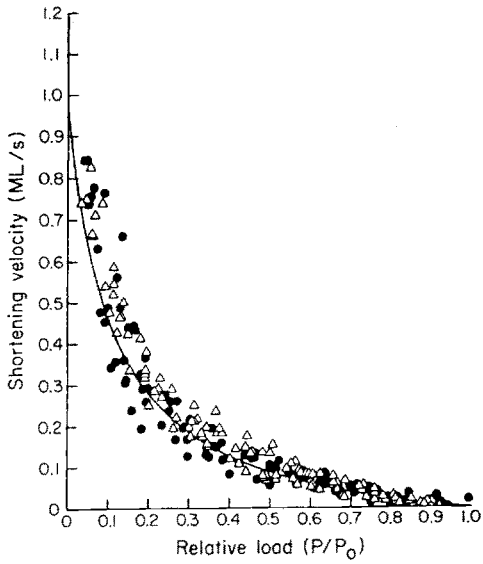

Fig. 5. Force-velocity data from small bundles of living soleus muscle fibers from control and aged rats. The fitted curve extrapolated to an unloaded shortening velocity of 0.97 muscle lengths/s. Data were obtained during tetanic stimulation at room temperature. (From Eddinger et al. 1986)

inversely with the size of the load. In intact muscle the shape of the force-velocity relationship is generally hyperbolic (Fig. 5) and can thus be described by the equation: $(P + a)(V + b) = \text{constant}$, in which a and b define the asymptotes of the hyperbola (Hill 1938; Katz 1939; Woledge et al. 1985).

While a hyperbola is usually a good approximation of the force-velocity relationship, velocity points at loads near zero or near maximum velocity deviate from a hyperbola: points near zero load generally lie above the hyperbola fitted to intermediate loads (e.g., Claflin and Faulkner 1989; earlier work discussed by Woledge et al. 1985), as do points at loads near the steady isometric tension (e.g., Edman 1988). Nonetheless, a fitted hyperbola provides a means for describing the curvature of the force-velocity relationship and extrapolation of the fitted curve to zero load provides an estimate of unloaded shortening velocity, V_{max}. If the load on a muscle is greater than P_0, the muscle actually lengthens at a low velocity (Katz 1939). Thus, the load that can be lowered by a muscle is considerably greater than the load that can be lifted or held isometrically. The velocity of lengthening increases gradually until the load is equivalent to about $2 \times P_0$, above which velocity increases rapidly. The basis for the sudden increase in velocity of lengthening is not known, but this mechanism presumably provides protection against muscle injury due to very large loads.

Mechanical V_{max} in a variety of skeletal muscles appears to be directly related to the V_{max} of actin-activated myosin ATPase activities

of proteins from the same muscles (Barany 1967). Based on a correlation between shortening velocity and rate of ADP release in a variety of muscles, Siemankowski et al. (1985) have suggested that maximum shortening velocity is limited by the rate of ADP dissociation from the cross-bridge which effectively controls detachment of the cross-bridge from actin. Consistent with this idea, Huxley's (1957) two-state model of contraction predicts that V_{max} is exquisitely sensitive to detachment rate since cross-bridges that remain bound after completing their power strokes ultimately become a load on the muscle as shortening continues and thereby limit V_{max}. In Huxley's model cross-bridges that are slow to detach (due to slow rates of ADP dissociation) ultimately become compressed as thick and thin filaments continue to slide past one another, giving rise to a restoring force that opposes contraction and slows velocity.

Maximum shortening velocity and the force-velocity relationship are frequently used to characterize functional differences in skeletal muscles due to alternate expression of myosin isoforms. When experimental conditions such as temperature and sarcomere length are equivalent, V_{max} provides an assessment of the relative rates of ADP release from different myosin isoforms complexed with actin; however, V_{max} can also vary with experimental conditions, and these must be taken into account when comparisons are made. In addition, while V_{max} and actin-activated ATPase activity are well correlated in muscles of different types and from different sources (Barany 1967), these properties should not necessarily be interpreted as probing the same transition in the cross-bridge interaction cycle, since many investigators believe that ATPase activity, which is a manifestation of cross-bridge turnover rate, is limited by the rate of ATP hydrolysis while the cross-bridge is dissociated from actin. Also, the rate of ADP dissociation during shortening is probably different than in isometric contractions, because the mean mechanical strain is less than in isometric contractions and many rate constants of the interaction cycle are strain dependent (Huxley 1957).

2.3.2 *Effects of* $[Ca^{2+}]$ *on Shortening Velocity*

The possibility that Ca^{2+} influences the velocity of muscle shortening was for many years a controversial idea, since some investigators found that reductions in $[Ca^{2+}]$ reduced V_{max} in skeletal muscle (Julian 1971) while others found no change (Teichholz and Podolsky 1970; Podolin and Ford 1986). In this regard Huxley's 1957 model predicted that activation would not affect $V_{max,}$ since V_{max} is not thought to vary with

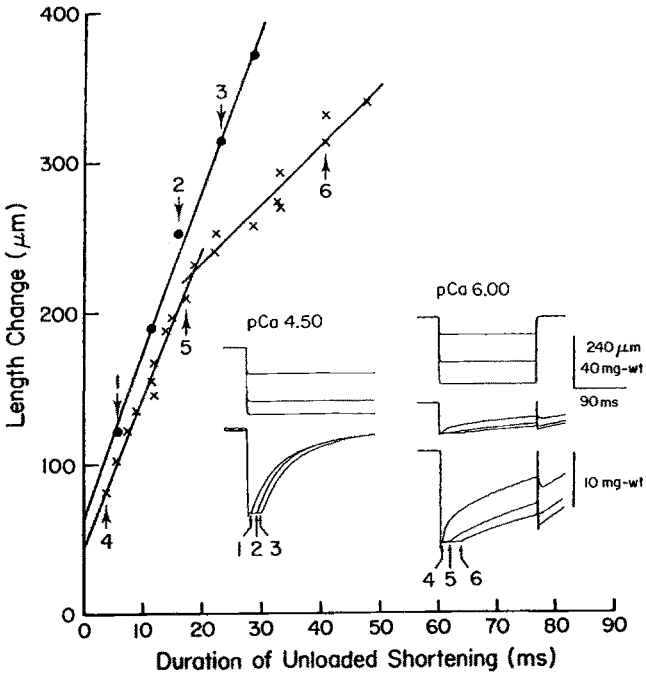

Fig. 6. Slack test plots from rabbit skinned psoas fiber showing the effect of level of activation on V_{max}. During maximal activation (●), V_{max} (indicated by the slope of the line fitted to the data points) is high and does not change with the extent of shortening. At submaximal activation (×), shortening is biphasic such that V_{max} is reduced for greater extents of shortening, i.e., there are high-velocity and low-velocity phases of shortening. As explained in the text, V_{max} in the low-velocity phase has greater dependence on level of activation, but in both cases, Vmax decreases if $[Ca^{2+}]$ is sufficiently reduced. Insets show original recordings of length (upper) and tension at each pCa. Numbers indicate time points at which tension began to redevelop and corresponding points on the plots. (From Moss 1986)

numbers of interacting cross-bridges but instead is determined by the rate of cross-bridge dissociation. When a muscle is shortening at V_{max}, the proportions of cross-bridges with strains greater than 1.0 and those with cross-bridge strains less than 1.0 would be equal, and external force developed by the muscle would be zero. More recent work (Moss 1986; Farrow et al. 1988) clearly shows that $[Ca^{2+}]$ has a profound effect on V_{max} and also provides an explanation for the earlier discrepancies. At levels of Ca^{2+} that were saturating with respect to isometric tension, shortening velocity is high and is constant during the period of shortening (Fig. 6). However, at submaximal levels of Ca^{2+} shortening is biphasic: there is an initial phase of high-velocity shortening followed by a linear phase of low-velocity shortening. V_{max} in the high velocity phase does not vary with $[Ca^{2+}]$ unless the isometric tension generating

capability of the fiber is less than 0.3 P_0. On the other hand, V_{max} in the low velocity phase decreases with [Ca^{2+}] over the entire range studied. Thus, in previous studies, if V_{max} was measured at moderate levels of Ca^{2+} (i.e., > 0.3 P_0) or soon after changes in load (Teichholz and Podolsky 1970; Podolin and Ford 1986), no effect of Ca^{2+} would be seen. However, if V_{max} was assessed at low levels of activation or at longer times after a change in load or length, a clear reduction would be evident. Based on biochemical results (Siemankowski et al. 1985) it seems likely that the reduction in high-velocity V_{max} at low levels of Ca^{2+} is due to slowing of ADP release.

The mechanism of activation-dependent changes in V_{max} is incompletely understood (Moss 1992), although most features of results to date can be explained by either of two models. In either scheme the Ca^{2+} sensitivity of the high-velocity phase is probably due to reduced rates of ADP dissociation at low [Ca^{2+}], which has been observed in proteins in solution (Rosenfeld and Taylor 1987). One possibility to explain the low-velocity phase of shortening (Moss 1986; Metzger and Moss 1988a) is that in partially activated fibers cross-bridges are bound to the thin filament in zones of transition between fully active and fully inactive functional groups. However, because of the partial blocking position of Tm, these cross-bridges are unable to attain a state (i.e., with no nucleotide bound) from which they could dissociate in the presence of ATP. As the muscle shortens, these cross-bridges eventually constitute an internal load that retards further shortening (Fig. 7). Low-velocity V_{max} would be expected to decrease as [Ca^{2+}] is reduced since the proportion of cross-bridges that are long-lived should increase. Alternatively, as the muscle shortens, the number of cross-bridges interacting with the thin filament decreases (Huxley 1957; Julian and Sollins 1975). Reductions in the number of strongly bound cross-bridges reduces the kinetics of interaction of cross-bridges with the thin filament (Swartz and Moss 1992) and thus might be expected to reduce shortening velocity. This second model does not provide a simple explanation for the two phases of shortening at low levels of activation, although it is possible that the number of strongly interacting cross-bridges decreases gradually as shortening proceeds, and that the state of activation of the thin filament is reduced only when the number of cross-bridges is less than a threshold value. If this were the case, shortening velocity would initially be high and would slow only when numbers of strongly bound cross-bridges are sufficiently reduced.

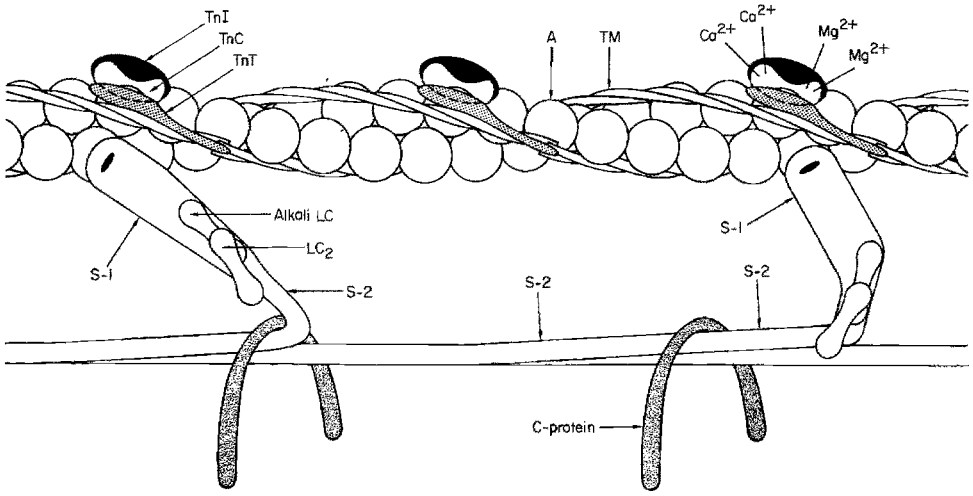

Fig. 7. Schematic representation of thick and thin filaments showing possible mechanisms for determining V_{max} in muscle and for slowing of V_{max} at low levels of activation. Left, Representation of a slowly cycling cross-bridge, proposed to exist at low levels of activation, that has been carried beyond the normal configuration as a result of shortening. Further sliding of the thick and thin filaments beyond this point would be impaired by stretch of S-2 and the overall rate of shortening would be slowed. Right, Cross-bridge in the normal force-generating configuration. If this cross-bridge is long-lived, continued shortening would result in a configuration similar to the left. However, if this is a normally cycling cross-bridge, further shortening causes compressive strain of S-2 and detachment from actin before S-2 buckles. In both cases, the accumulation of compressively strained or buckled cross-bridges presumably depends on the rate of ADP dissociation from the actin/mysoin-ADP complex prior to the cross-bridge detachment step. (From Hofmann et al. 1991a)

2.3.3 Power Versus Load Curves

In performing work against a load muscles generate power, which is work per unit time and is obtained by multiplying force × velocity. Since force and velocity are inversely related, the product of the two is greatest at an intermediate load, which turns out to be about 0.3 P_0. At this peak of the power curve, skeletal muscle is working most efficiently since 40%–45% of the chemical energy is converted to mechanical work.

Several investigators have characterized the ability of muscles to perform work by transforming force-velocity data to power-load curves. Because power reaches a maximum in the middle ranges of both force and velocity in muscles, and velocity varies rather slowly with load in this range, some investigators (Brooks et al. 1990; Seow and Ford 1991) have suggested that maximum power output is a more reliable index of dynamic mechanical properties of a muscle than is V_{max}. Since V_{max} is

usually estimated by extrapolating the steep part of the force-velocity curve to zero load, small errors in estimating loads near zero can have dramatic effects on estimates of values for V_{max}. In addition, maximum power provides a measure of mechanical properties of muscles operating under physiological loads. Muscles in vivo do not generally operate at either extreme of the force-velocity curve, i.e., at V_{max} or 0, where power is zero, but instead operate at intermediate forces and velocities where power is close to its maximum.

Brooks et al. (1990) measured maximum power outputs from fast-twitch and slow-twitch muscles of mice and determined that while isometric tension does not differ between the two types of muscles, fast-twitch muscle has significantly greater maximum power output than does slow-twitch muscle. Further studies have found the relative velocity (V/V_{max}) at which maximum power is attained to be a key parameter in defining the mechanical properties of a muscle. Rome et al. (1988) determined that in swimming carp, both slow and fast muscles operate at approximately the same V/V_{max}, although the absolute velocities differ considerably in the two muscle types. Rome and coworkers further determined that this value of V/V_{max} corresponds to the velocity at which maximum power is generated. Subsequent studies (Rome et al. 1990; Rome and Sosnicki 1990) have indicated that muscles operate within the same narrow range of V/V_{max}, independently of temperature, suggesting that this ratio is an important constraint in defining the operation of muscles in vivo. Such a constraint raises the possibility that changes in muscle contractile proteins that are observed during development or in response to changing functional demands may be an adaptive response to shift shortening velocity so that the muscle continues to operate in the range of V/V_{max} at which power output is maximal.

To date, power-load curves have not been extensively characterized in any muscle type with respect to possible sensitivity of peak power to Ca^{2+}, pH or other determinants of shortening velocity. Ford et al. (1991) investigated possible changes in normalized peak power with changes in $[Ca^{2+}]$ and found the peak to be unaltered when activation was lowered from P_0 to approximately 0.5 P_0 by reducing $[Ca^{2+}]$. This result suggests that the maximum rate at which a muscle can perform work does not vary with $[Ca^{2+}]$, at least when changes in tension generating capability are taken into account. However, based on previous measurements of Ca^{2+} sensitivity of V_{max} firm conclusions regarding possible Ca^{2+} sensitivity of peak power must await measurements of power at still lower concentrations of Ca^{2+} and at longer times following imposition of load.

2.4 Tension Transients

Tension transients in response to mechanical perturbations and rapid changes in solution composition provide information about specific transitions in the cross-bridge interaction cycle. Transients have provided important information regarding mechanisms of contraction, but the reader should also be aware that measurements from force transients are subject to artifact if there is significant compliance at the points of attachment to the experimental apparatus. If the ends are compliant, rates of rise of tension are slowed as the compliant ends are extended by the actively contracting middle region of the fiber (see review by Moss 1992). For this reason assessment of tension transients is best conducted in a mechanical system that allows monitoring and precise control of sarcomere length during the mechanical measurement. Several approaches have been used in studies of tension transients.

2.4.1 Transients in Response to Rapid Changes in Length

Following rapid reductions in length complete within 1 ms, muscle fibers exhibit isometric force transients (Fig. 8) that include a phase that is thought to represent the force-generating transition(s) of the cross-bridge interaction cycle (Huxley and Simmons 1971; Ford et al. 1977). When a step decrease in length is applied to a muscle fiber, tension decreases by an amount that is proportional to the length change, suggesting that the reduction in tension is due to recoil of elastic structures in the cross-bridge. This phase of the transient is followed by a phase of rapid recovery of force in which cross-bridges remain strongly bound to actin and presumably undergo mechanical and/or chemical transitions to higher force-generating states. Recent evidence suggests that this phase of tension recovery is coincident with a conformational change of the cross-bridge inferred from high time resolution X-ray diffraction patterns obtained during the transients (Irving et al. 1992). The third phase of recovery is a transient pause, plateau, or reversal in recovery, which is believed to be due to cross-bridge detachment. In the final slow phase of recovery cross-bridges bind or rebind, and force attains a new steady-state level.

If the length step applied to the muscle is sufficiently rapid (approximately 0.2 ms), it is possible to record phase 1 of the transient with minimal contributions from phase 2, the phase of rapid recovery (Ford et al. 1977). Assessment of phase 1 under these conditions suggests that elastic elements of the cross-bridge are strained approximately 4–8 nm

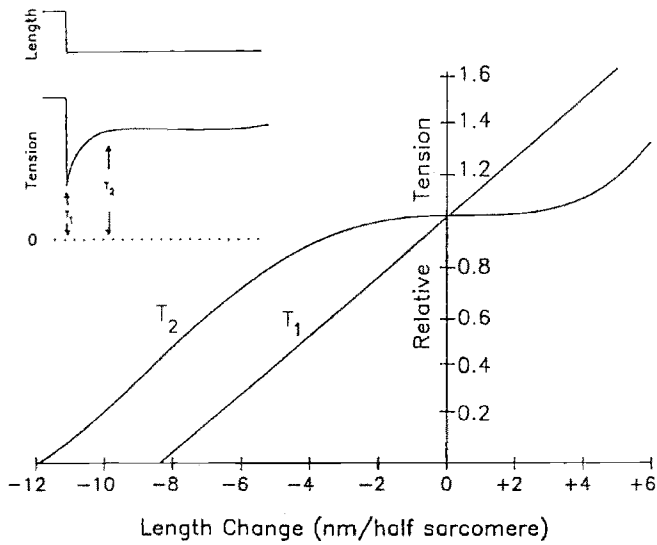

Fig. 8. Tension transient following rapid change in length of a single muscle fiber. Coincident with length change, tension decreases to a minimum (T1) which represents recoil of an elastic part of the cross-bridge due to relative sliding of thick and thin filaments. Once the length change is complete, tension recovers to an intermediate value, T2, without detachment of the cross-bridge from actin. This phase of force recovery is thought to be due to rotation of cross-bridge heads. Thus, the total working distance of a cross-bridge without detaching and reattaching is equivalent to the smallest imposed length changes (approximately 12 nm/half-sarcomere) for which T2 is zero. (Idealized diagram based on data from Ford et al. 1977)

in the process of isometric force generation. The distance over which myosin interacts with actin during a single cycle is more than this amount, since rapid recovery to phase 3 is observed even when a step decrease of approximately 10 nm is applied to the fiber. To date there is no evidence that the working stroke or interaction distance of cross-bridges varies with myofibrillar protein composition, although it is likely that the rates of tension change in various phases of the isometric tension transients are myosin isoform dependent.

Recent evidence suggests that the distance over which myosin and actin interact is much greater than that suggested by the work of Huxley and colleagues. For example, Higuchi and Goldman (1991) put single fibers into rigor, then rapidly generated various concentrations of Mg-ATP by photolysis of chemically caged ATP (see Sect. 2.4.2), and finally allowed the fibers to shorten under small loads until the ATP was consumed and shortening ceased. Knowing the concentrations of myosin and free ATP, the percentage of cross-bridges interacting with actin, and the distance shortened, they calculated an interaction distance of 60 nm or more for each molecule of ATP that was hydrolyzed. Because this

distance is much greater than the length of the myosin head (approximately 19 nm), they proposed that this long interaction distance involves a number of cycles of attachment and detachment, each of which is accompanied by a force-generating ministroke of the cross-bridges. Strong evidence for such a possibility was obtained in mechanical experiments in which muscle fibers took only 15 ms to completely regenerate their ability to undergo a cross-bridge power stroke (Lombardi et al. 1992). A length step applied a few milliseconds after an initial length step resulted in incomplete recovery of force during the fast phase; however, steps applied 15 ms or more after the first step resulted in full recovery of tension during the fast phase. This time-course of regeneration is very fast compared to the overall cycling rate of 1–3 s^{-1} for the actomyosin ATPase and suggests that cross-bridges are capable of a number of power strokes per ATP hydrolyzed.

In vitro motility assays have been especially valuable in studies of the working distance of cross-bridges per molecule of ATP that is hydrolyzed (Huxley 1990). In these assays (Kron et al. 1991) myosin or its subfragments are placed on a coverslip and become adherent to the glass surface. Droplets of solution containing fluorescently labeled thin filaments are then superfused over the coverslip, and motion of the thin filaments is then recorded using fluorescence microscopy and video technology. The theory of the measurement of the working distance of a cross-bridge is explained in detail by Huxley (1990): calculation of working distance requires knowledge of the ATPase activity per unit length of total actin on the coverslip and the velocity of movement of the thin filaments and an assumption of the number of cross-bridges bound per unit length of thin filament. To date studies involving in vitro motility assays have yielded widely disparate values for the working distance of the cross-bridge, ranging from 5–20 nm/ATP (Uyeda et al. 1991) to as great as 200 nm/ATP (discussed by Ishijima et al. 1991). We are not aware that this approach has yet been used to study possible differences in the sliding velocities or fundamental working distances of various myosins from mammalian striated muscles.

2.4.2 Transients Following Photolysis of Caged Compounds

In investigating molecular mechanisms by which protein isoforms influence contractile properties of muscle, rapid changes in solution composition would provide a means to probe specific transitions within the cross-bridge interaction cycle. For example, by rapidly changing the concentration of P_i it is possible to assess the kinetics of cross-bridge tran-

sitions associated with force development. These kinds of experiments have already been perfomed in fast-twitch muscle fibers of the rabbit by rapidly releasing compounds of interest from chemical cages which make the compounds inert and therefore invisible to the contractile proteins (Homsher and Millar 1990; Kaplan 1990). Ca^{2+}, P_i, and nucleotides such as ATP and ADP can be chemically caged as part of photolabile compounds. Exposing these compounds in solution to bright light (λ = 347 nm) results in photolysis of the chemical cage and release of the compound of interest. In this way sudden increases in the concentration of a variety of compounds can be achieved, thereby perturbing the transition in the cycle in which the compound participates as a reactant or product. Photorelease of compounds is accompanied in every instance by a mechanical force transient, which allows inferences about the role of the underlying transition in force generation. Again using P_i as an example, caged compounds have been used to determine that P_i release is associated with force development in muscle (Homsher and Millar 1990), that P_i release is a two-step process involving an isomerization of the actomyosin-nucleotide complex either before or after P_i release (Millar and Homsher 1992; Dantzig et al. 1992; Walker et al. 1992), and that the rate of P_i release is cross-bridge strain dependent (Homsher and Lacktis 1988) and Ca^{2+} sensitive (Walker et al. 1992). Numerous experiments have been carried out to probe other aspects of the cross-bridge interaction cycle, including the ADP release step using caged ADP (Dantzig et al. 1992; Lu et al. 1993), dissociation of rigor cross-bridges from actin using caged ATP (Goldman et al. 1984a,b), and the rates of activation and relaxation of skinned muscle fibers using caged Ca^{2+} (eg, Lea et al. 1990) and caged chelators of Ca^{2+} (e.g., Lannergren and Arner 1992). Despite the impressive results that this general approach has yielded in fast-twitch muscle fibers, no studies published to date have used flash photolysis of caged compounds to compare the kinetics of specific cross-bridge state transitions in fibers having widely divergent protein isoform compositions.

2.4.3 Tension Redevelopment Following Release and Restretch

The rate at which force is developed during the onset of contraction is considerably faster in fast-twitch than in slow-twitch muscles. The faster rate could be due to faster delivery of Ca^{2+} to the myoplasm during excitation-contraction coupling and/or to faster transitions of cross-bridges to force generating states once Ca^{2+} is bound by the thin filaments. One way to distinguish between these possibilities is to attempt

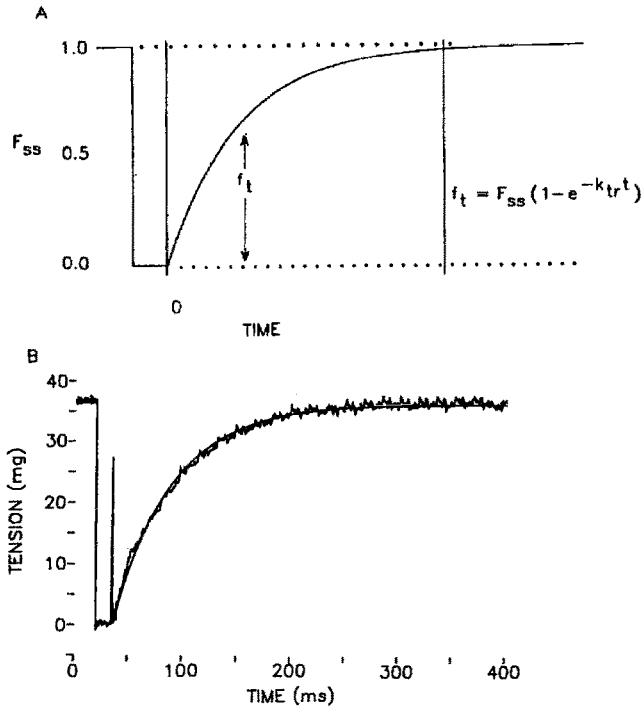

Fig. 9. Determination of k_{tr} in rabbit skinned psoas fibers. Schematic diagram of an experimental record showing the measured variables and the equation used for determining k_{tr}. **A** Once a fiber was steadily activated in a Ca^{2+}-containing solution and tension was constant (*ss*), the fiber was slackened and tension was reduced to zero. Following a period of unloaded shortening, the fiber was rapidly reextended to its original length, thereby straining attached cross-bridges and transiently increasing tension. The strained cross-bridges then rapidly dissociated from actin, reducing tension to zero. The subsequent time-course of tension recovery represents the redistribution of cross-bridges from non-tension-generating to tension-generating states. **B** Actual record obtained during an experimental measurement of k_{tr} at 15°C. line, computer-fitted curve for which k_{tr} was 18 s^{-1}. Sarcomere length was kept constant (± 0.5 nm) by controlling the position of the first-order line of a laser diffraction pattern obtained from the fiber. (From Metzger et al. 1989)

to change $[Ca^{2+}]$ rapidly in the vicinity of the myofilaments in skinned fibers (see Sect. 2.4.2) and then record the rate of tension development. An alternative approach is to maintain the skinned fiber in a steady Ca^{2+}-activated state and then apply a mechanical perturbation that disrupts formed cross-bridges and reduces force to near zero. The subsequent redevelopment of force would then represent the transition from non-force-generating to force-generating states without limitations due to diffusion of Ca^{2+}, as would be expected in excitation-contraction coupling in living fibers. Brenner (1986) has developed a very effective protocol to study the force generating transition in steadily Ca^{2+}

activated skinned fibers (Fig. 9): fibers are released, allowed to shorten under no load for a distance that requires several cycles of interaction of cross-bridges with actin, and then restretched to the original length to break most of the cross-bridges still bound to actin. Force at the end of restretch is zero or nearly zero since all or most cross-bridges are in detached, weakly bound states. The subsequent recovery of active force results from the transition of weakly bound cross-bridges into strongly bound, force-generating states. In most cases the record of force redevelopment can be fit with a single exponential of the form, tension = $C(1-e^{-kt})$, in which C is steady isometric force, and k is the rate constant of force redevelopment, k_{tr}. Using a simple two-state model for cross-bridge interaction, A + M $\leftrightarrow$ AM, in which the formation of the force-generating AM complex is regulated by rate constant f, and dissociation of the complex is regulated by rate constant g, Brenner and Eisenberg (1986) assumed that k_{tr} was the sum of f and g. This is an especially useful way to interpret k_{tr} since force and ATPase activity can also be expressed in terms of these rate constants. Assuming this model, it is then possible by measuring combinations of force, k_{tr} and ATP activity to assess the effects of various experimental perturbations on one or both rate constants. For example, measurements that showed k_{tr} is Ca^{2+} dependent have been interpreted as showing that Ca^{2+} has this effect primarily by altering f, the rate constant of the non-force- to force-generating transition(s).

Of course, a simple two-state cross-bridge model does not account for all experimental results. For example, the Ca^{2+} sensitivity of the force-generating transition inferred by Brenner implies that k_{tr} is P_i dependent, which it is (Walker et al. 1992), and that the rate constant of tension change (P_i) upon photolysis of caged P_i varies with $[Ca^{2+}]$, which it does (Walker et al. 1992). However, P_i shows only a threefold sensitivity to Ca^{2+}, which is not sufficient to account for the approximately 20-fold regulation of ATPase activity during activation of contraction (Levy et al. 1976); also, in any case, k_{tr} is substantially smaller than P_i suggesting that k_{tr} is dominated by a process not probed by P_i. Homsher and Millar (1990) proposed that during the measurement of k_{tr}, dissociation of strongly bound cross-bridges from actin results in cooperative inactivation of the thin filament, which is not the case in measurements of P_i. Thus, the lower value of k_{tr} might be due to cooperative reactivation of thin filament as strongly bound cross-bridges reform. This idea was tested in experiments in which k_{tr} was measured before and after application of an analog of strongly bound cross-bridges to skinned skeletal muscle fibers (Swartz and Moss 1992).

N-ethylmaleimide conjugated myosin subfragment 1 (NEM-S1) binds strongly to thin filaments and has previously been shown to cooperatively activate actin-activated ATPase activity (Williams et al. 1988). Application of NEM-S1 to skinned fibers increased k_{tr} to maximal values even at very low concentrations of Ca^{2+} (Swartz and Moss 1992), suggesting that strongly bound cross-bridges activate kinetic transitions in the cross-bridge interaction cycle. On the other hand, even in the presence of NEM-S1 the maximum value of k_{tr} was no greater than that obtained in the presence of saturating [Ca^{2+}]. Thus, the basis for differences between maximum values of k_{tr} and k_{Pi} are still uncertain, although it seems likely that a step prior to force development, such as cross-bridge binding to the thin filament, limits k_{tr} but not k_{Pi}.

3 Mechanical Effects Due to Natural Transitions in Isoform Content in Mammalian and Avian Muscles

3.1 Myosin Heavy Chains

3.1.1 Subunit Structure of Myosin

The contractile protein myosin is a hexamer comprised of two heavy-chain subunits of approximately 220 kDa mol. wt. and four light-chain subunits of approximately 20 kDa each. MHCs and MLCs are both encoded by a highly conserved multigene family and are expressed in muscle as multiple isoforms. The MHCs found in the myofibrils of mammalian striated muscles are all isoforms of myosin family II, and to date as many as ten isoforms have been identified. In striated muscles of the rat, which is probably the most extensively studied species with respect to MHC diversity, the following MHCs have been described: cardiac α and β, skeletal slow type I (which is the same gene product as cardiac β), embryonic, neonatal, and three fast MHCs–types IIa, IIb and IIx (or IId). A detailed description of the MHC and MLC families can be found in extensive reviews by Swynghedauw (1986), Pette and Staron (1990), and Bandman (1985, 1992). Rather than reiterate points made in earlier reviews, the present description focuses on possible relationships between the contractile properties of muscle fibers and contractile protein isoforms. Since myosin is the most abundant of the myofibrillar proteins, and since it contains both the actin binding site and the myosin ATPase site, isoforms of MHC have figured prominently in studies of determinants of contractile properties in muscle.

Skeletal muscles have generally been categorized on the basis of contraction speed in terms of either velocity of shortening or twitch kinetics: muscles with slow contractile properties typically express a preponderance of slow-type MHCs, while fast muscles usually contain mostly fast-type MHCs (Pette and Staron 1990). This correlation between contractile properties and MHC isoform distribution strongly suggests that MHC isoforms play an important role in determining contractile properties of single muscle fibers. In turn, the contractile properties of a whole muscle result from the aggregate properties and myosin isoform content of its constituent fibers.

3.1.2 Variation of Twitch Kinetics with Myosin Heavy Chain Content

Contraction time, i.e., time to peak tension, one-half relaxation time, or twitch duration, has been widely used in distinguishing between muscle types. As mentioned above, muscles with faster contraction times generally contain a greater percentage of fast myosin isoforms (Staron and Pette 1987; Termin et al. 1989a,b), and across species faster contraction and half-relaxation times are associated with fast fiber types (Heizmann et al. 1982). However, we are not aware of studies that quantitatively relate twitch contraction times with MHC content. As muscles develop during maturation, twitch contraction time decreases, and this correlates with a reduction in the proportion of slow myosin, as detected by immunoreactivity to slow myosin antibodies (Gauthier et al. 1978). One ambiguity in the interpretation of correlations such as these is that contraction time is affected not only by the kinetics of actomyosin interaction but also by the time course of Ca^{2+} release and reuptake by the sarcoplasmic reticulum and the kinetics of Ca^{2+} binding to the thin filament.

In order to focus more directly on possible contributions of myosin isoforms to the rate of tension development during a twitch, Metzger and Moss (1990b) assessed the fiber type dependence of k_{tr}, the rate constant of tension redevelopment following a rapid release and restretch maneuver (Brenner 1986; Brenner and Eisenberg 1986). In maximally activated fibers k_{tr} was eightfold greater in fast-twitch fibers than in slow-twitch fibers identified on the basis of their MHC and alkali light-chain compositions. Thus, the transition to the force generating state is influenced by the type of MHC present. At lower levels of Ca^{2+} activation, there was only a threefold difference in k_{tr}, further suggesting that the activation state of the thin filament influences the rate of tension development differently in fast-twitch and slow-twitch fibers.

3.1.3 Variation of Shortening Velocity with Myosin Heavy Chain Content

Mechanical V_{max} and force-velocity relationships are commonly used to classify muscles as slow or fast. In tetanically stimulated muscles, measurements of V_{max} are presumably not influenced by the dynamics of Ca^{2+} cycling that define the twitch, since V_{max} is measured only after the muscle has reached complete, steady activation. Thus, it seems likely that variations in V_{max} among tetanically stimulated whole muscles (Barany 1967; Close 1972b) are due primarily to differences in relative proportions of MHCs or MLCs. Studies to date have shown that myosins isolated from slow muscles have lower ATPase activities than myosins from fast muscles (Sreter et al. 1966; Thomason et al. 1986; Tsika et al. 1987a). However, beyond these general observations and conclusions it is difficult to ascribe particular mechanical behaviors to specific combinations of MHC or MLC isoforms. Ambiguity arises due to variability between fibers in expression of myosin isoforms, so that whole muscle behavior represents some mean of the contractile properties of individual fibers that comprise the muscle. To date there have been relatively few studies that have directly measured shortening velocity and myosin isoform compositions in the same single muscle fibers. Those that have been published have focused primarily on the roles of MHC isoforms in determining V_{max}, a rationale that no doubt derived in part from earlier biochemical observations that removal of MLCs has little effect on actin-activated myosin ATPase activity in solution (Sivaramakrishnan and Burke 1982; Wagner and Giniger 1981; Wagner and Weeds 1977).

Reiser et al. (1985a) provided some of the first evidence of a relationship between V_{max} and MHC isoform content in mammalian striated muscle, i.e., rabbit soleus muscle. Rabbit soleus is thought to be virtually entirely comprised of slow fibers. In this study, however, a small number of fibers were found in which V_{max} was significantly greater than the mean V_{max} of the majority of fibers from these muscles. The faster fibers were found to contain a mixture of both fast MHC and slow MHC, and V_{max} was found to increase linearly as the proportion of fast MHC increased (Fig. 10).

Several studies have taken advantage of natural transitions in MHC isoforms during development and maturation to determine the influence of the MHC isoforms on shortening velocity. During development, the mechanical properties of rabbit psoas muscle fibers gradually change from slow to fast. At the 7-day stage of postnatal development a wide range of shortening velocities was detected among single fibers from psoas muscles (Reiser et al. 1985b). The 7-day stage is the time point in

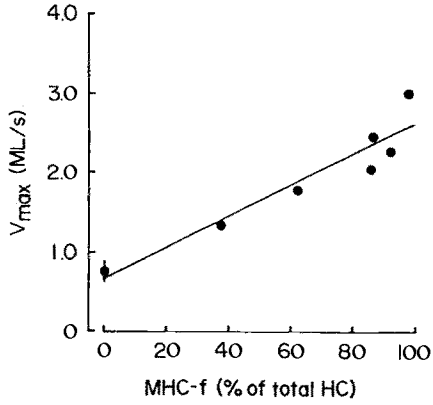

Fig. 10. Plot of V_{max} versus fast MHC content (as percentage of total MHC) of single fibers from rabbit soleus fibers. V_{max} was determined using the slack test, and MHC content was determined from densitometric scans of SDS polyacrylamide gels of the fibers on which mechanical measurements were made. In each case, the remainder of MHC content to a total of 100% was comprised of slow (type I) MHC. (From Reiser et al. 1985a)

development at which there was the widest range in the mixture of two MHC isoforms expressed in this muscle. At times both before and after the 7-day stage there was both a narrower range of shortening velocities and less variation in expression of MHC isoforms. In another study Reiser et al. (1988b) determined V_{max} and MHC composition in single fibers from the slow anterior latissimus dorsi (ALD) muscle of chickens at both neonatal and adult stages of development. Avian muscle expresses two slow-type MHCs (SM1 and SM2), and in the ALD the relative percentage of SM2 increases during maturation. A highly significant correlation was found between the relative proportions of SM1 and V_{max} of single ALD fibers at both the neonatal and adult stages. Only slow-type MLCs were found in the ALD at these stages, and thus the authors concluded that the MHC composition of avian ALD muscle fibers is the primary determinant of $V_{max.}$. A similarly significant correlation was found between mechanical V_{max} and the percentage of adult fast MHC in single fibers from the developing rat soleus muscle (Reiser et al. 1988a).

A recent study by Bottinelli et al. (1991) used a series of monoclonal antibodies to more accurately detect the presence of each of the MHC isoforms. Mechanical properties were determined in single fibers taken from three different muscles of the rat, and the fibers were then classified as either type I, IIA, IIB, or IIX based on their reactivity to the antibodies. The muscle fibers that these authors studied formed a continuum with respect to shortening velocities, which ranged from 0.35 to 2.84 muscle lengths per second (Fig. 11). The type I fibers had the lowest shortening velocities and comprised a discrete group. However, there was considerable overlap among all of the type II fibers, although the mean V_{max} of type IIB fibers was significantly higher than that for the

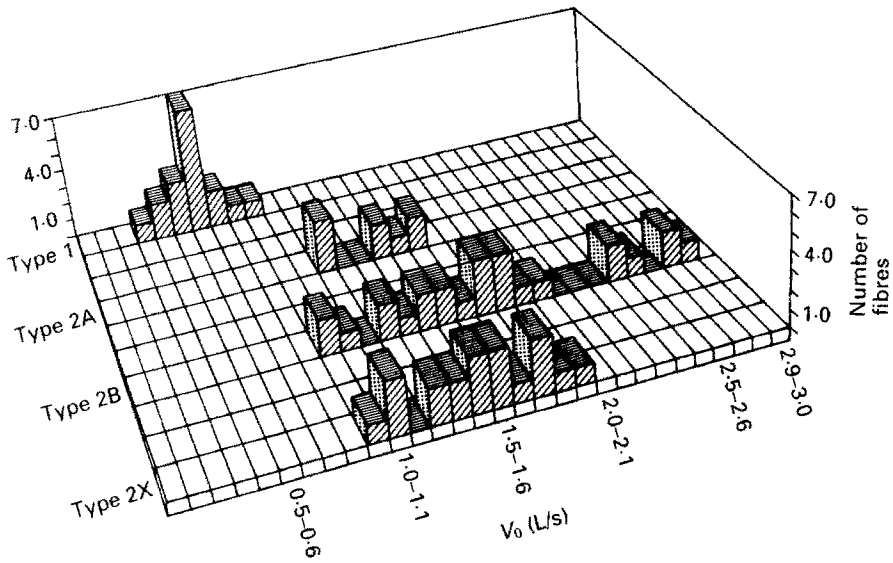

Fig. 11. Distributions of maximum shortening velocities measured in four types of fibers from rat skeletal muscles. Fiber types were identified with monoclonal antibodies to each MHC isoform. (From Bottinelli et al. 1991)

type IIA and IIX fibers. In a more recent study Galler et al. (1994) found that a large proportion of fibers from rat leg muscles coexpressed two or more MHC isoforms. There was a wide range of shortening velocities among these fibers and large degrees of overlap in shortening velocities among fibers expressing a single MHC isoform and fibers expressing two or more type II MHC isoforms. These investigators (Bottinelli et al. 1991; Galler et al. 1994) concluded that MHC content is a significant determinant of shortening velocity, but because of the overlap they observed they also proposed that other factors may be important in determining variations in shortening velocities among fibers having identical MHC content.

Sweeney et al. (1988) also reported variations in maximum shortening velocities among fibers having the same MHC composition. Fibers from rabbit psoas muscle had significantly higher V_{max} values than fibers from rabbit tibialis anterior, but the MHC compositions of these two groups of fibers as determined from peptide maps were indistinguishable. However, differences in shortening velocity between these two groups of fibers were correlated with differences in alkali light chain content. There was a greater variation in shortening velocity between fibers of different MHC composition (IIa versus IIb) than among fibers with the same (IIb) MHC content. These authors concluded that variations in shortening velocity between IIA and IIB fibers could be

explained by differences in MHC composition, while differences among the IIB fibers were attributed to differences in alkali light chain content. In this regard, Wada and Pette (1993) reported a wide variation in alkali light content in rat and rabbit fibers having identical MHC composition.

Variability in shortening velocities among fibers with the same MHC composition might also be related to the presence of undetected MHC isoforms in these fibers. Fast (type II) MHC isoforms are particularly difficult to distinguish, and multiple expression of type II isoforms within the same single fiber may therefore be easily overlooked. Early studies of mechanical properties of single fibers in relation to MHC content reported only the presence of fast and slow MHCs, without differentiating among the various type II MHC isoforms (Reiser et al. 1985a). Even the most sensitive detection methods, such as the immunohistochemical approach used by Bottinelli et al. (1991), may not resolve small amounts of a particular type II MHC isoform that is coexpressed with another, more predominant type II isoform in the same fiber. Variability in contractile properties in fibers having apparently similar MHC composition might also be due to the presence of other, as yet unidentified MHC isoforms. As an example of this idea, the existence of a third type of type II MHC, i.e., IIa/d, has only recently been discovered (Schiaffino et al. 1989; Termin et al. 1989a), and the number of identified MHC isoforms continues to grow (see Hughes et al. 1993 for a description of three slow MHC). In this regard, Robbins et al. (1986) have indicated that in the chicken there are as many as 31 genes within the MHC family. Thus, ultimate understanding of the roles played by MHC isoforms in determining contractile properties will require quantitative mechanical measurements in combination with detection techniques sufficiently sensitive to describe the MHC family.

With regard to Ca^{2+} sensitivity or activation dependence of V_{max}, it is evident from studies on rabbit chemically skinned single fibers that V_{max} in both fast-twitch (type IIB) and slow-twitch (type I) fibers decreases when the level of activation is reduced (Moss 1986; Metzger and Moss 1988a). This phenomenon has not been studied in sufficient depth to determine whether the magnitude of effects or sensitivity to Ca^{2+} are similar in the two types of muscle. Nor is it known whether isoforms of MHCs confer different sensitivities of actomyosin interaction kinetics to level of thin filament activation. It is possible, for example, that the thin filament alone confers sensitivity of V_{max} to level of activation.

3.2 Alkali Light Chains

From the discussion of possible roles of MHC isoforms in determining V_{max}, there is a considerable amount of unexplained variability in V_{max} among fibers that appear to contain similar MHC isoforms. As referenced in Sect. 3.1, several investigators have proposed that fiber-to-fiber variations in alkali light chain composition many contribute to the variability in V_{max}. There is in fact some evidence that this is the case, which is discussed below, but we first present general background concerning the alkali light chains.

Each myosin head has two associated light chains – a P-light chain, which is also called the regulatory light chain or light chain-2, and an alkali light chain, also called the essential light chain. Alkali light chains are so named because of a requirement for alkaline pH in the protocol for its extraction from MHC (Lowey and Risby 1971). In adult skeletal muscles, there are two fast-type alkali light chains (LC_1 and LC_3) and two slow-type (LC_{1aS} and LC_{1bS}) that are distinguished by differences in apparent molecular weight on sodium dodecyl sulfate (SDS) gels. The ratio of the two alkali light chains in each muscle type can vary considerably in developing and adult muscle, although the ratio of LC_1 to LC_3 in adult rabbit muscles is approximately 2:1. There is also an embryonic form of skeletal LC_1, called LC_{1e}, which is expressed in both fast-twitch and slow-twitch muscles during early stages of development. Adult cardiac muscle expresses alkali light chains as either ventricular (LC_{1v}) or atrial (LC_{1a}) isoforms, which is of some interest here since gene sequencing has shown that mouse skeletal LC_{1e} is equivalent to adult LC_{1a} and embryonic LC_{1v} (Barton et al. 1988); also, adult mouse LC_{1v} is identical to skeletal LC_{1bs} (Barton et al. 1985). These variations in light chain expression between and within muscle types may provide another potential avenue for adaptation of muscle to chronically altered work loads.

The roles played by the alkali light chains of myosin in muscle contraction is not well understood. Recent X-ray studies (Rayment et al.1993) of the alkali light chain, as well as the P-light chain, in association with myosin subfragment 1 (S1) yielded potentially important structural information about the light chains. The alkali light chain and to a lesser degree the P-light chain were found in close association with the α-helical portion of S1, suggesting the possibility that one or both of these light chains provide mechanical stabilization of the myosin head during force development. Other evidence suggests that the alkali light chains influence the rate of interaction of myosin

with actin, which implies that the light chain has a role in addition to mechanically stabilizing S1. For example, during maturation of mammalian fast-twitch skeletal muscle, the proportions of myosin alkali light chains change from virtually 100% LC_1 at birth to approximately 67% LC_1 and 33% LC_3 in the adult (Roy et al. 1979). During this same time the velocity of unloaded shortening (V_{max}) and the actin-activated myosin ATPase activity increase. However, since the MHCs are also changing during this time frame, variations in light chain content are unlikely to be the sole determinants of the increase incontraction speed.

The results of a few studies combining mechanical and biochemical approaches strongly suggest that there is a close association between V_{max} and natural variations in alkali light chain content of fast-twitch muscle fibers. For example, as described in Sect. 3.1, Sweeney et al. (1986, 1988) concluded that the variability in V_{max} measured in rabbit skeletal muscle fibers that appeared to contain only IIb myosin was due to variations in the proportions of LC_1 and LC_3. In another study (Greaser et al. 1988), V_{max} and myosin isoform composition were measured in plantaris muscles of the rabbit. In both cases V_{max} increased as the proportion of LC_3 increased, which is consistent with the previously observed association between increases in LC_3 and V_{max} during development. However, there are potential complications in both of these studies on skinned fibers that make it difficult to firmly conclude that there is a causal relationship between changes in alkali light chain composition and V_{max}. With respect to the studies on plantaris fibers, the content of isoforms of fast TnT varied consistently with LC3 content and thus could have had an effect on V_{max}. Also, Sweeney et al. (1988) observed differences in the peptide maps of MHCs from type IIA and IIB fibers of rabbit plantaris muscles, which suggests that in the present study there may be undetected differences in MHC composition among fibers in the study by Greaser et al. (1988). On the other hand, some variation in the V_{max} of IIB fibers in the study by Sweeney et al. may have been due to undetected presence of other isoforms of MHC. Other studies that have attempted to address this issue (Botinelli et al. 1991; Larsson and Moss 1993) have not found strong relationships between variations in V_{max} and alkali light chain composition, but the studies could not rule out the possibility that alkali light chains have a role in defining interaction kinetics. One way to address the experimental ambiguities of these previous studies is to directly manipulate the alkali light chain composition in a given single fiber so that MHC, P-light chain, and regulatory protein compositions are held constant (see Sect. 4.3).

Increases in V_{max} with changes in alkali light chain composition, such as those reported by Sweeney et al. (1986, 1988) and Greaser et al. (1988) are unexpected on the basis of solution biochemical work with LC_1 and LC_3 homodimers of intact myosin, in that actin-activated myosin ATPase activity was similar regardless of the alkali light chain that was present. Also, as described in Sect. 3.1, removal of the light chains did not appear to affect ATPase activity. The finding that velocity may be affected by alkali light chain composition but ATPase activity is not has led Lowey et al. (1993) to conclude that the light chains are important in force generation and movement in intact systems, but they do not play a significant role in modulating ATPase activity in solution (results that led to this conclusion are discussed in Sect. 4.3).

3.3 Thin Filament Regulatory Proteins

Both tension and maximum velocity of shortening are regulated by Ca^{2+} in vertebrate striated muscles. As discussed in Sects. 2.1 and 2.3, the Ca^{2+} dependence of these mechanical properties involves the binding of Ca^{2+} to low-affinity sites on TnC; however, Ca^{2+} sensitivity is also modulated by several physical factors (Sect. 2.1). In addition, it is increasingly clear that strongly bound cross-bridges and various isoforms of thin filament-associated regulatory proteins also influence the apparent binding affinity of TnC for Ca^{2+}, which are the main topics of this section.

3.3.1 Cooperative Activation of Tension

Tension-pCa relationships measured in skeletal and cardiac muscles are usually asymmetric in that they are steeper at low [Ca^{2+}] than at high. The steepness evident at low [Ca^{2+}] has generally been interpreted to mean that activation of contraction involves a high degree of molecular cooperativity among subunits of the thick and thin filaments, an inference that is supported by data from a variety of sources. From biochemical studies of myosin, regulated actin and micromolar Mg-ATP in solution, rigor cross-bridges bound to the thin filament appear to activate the filament to allow interaction with nucleotide-bound cross-bridges (Bremel and Weber 1972). Consistent with this idea, skinned fibers develop substantial tensions in 1 μ Mg-ATP even when Ca^{2+} is absent (Kawai and Brandt 1976) and slowly shorten under these same conditions (Moss and Haworth 1984). Brandt et al. (1987) suggested

that the steepness of the tension-pCa relationship at millimolar Mg-ATP actually involves sustained activation of regions of the thin filament due to bound cross-bridges, which have much longer half-times for dissociation from actin than does Ca^{2+} from TnC. The shape of the tension-pCa relationship may also be influenced by marked cooperativity in Ca^{2+} binding to the thin filament. Grabarek et al. (1983) found independent binding of Ca^{2+} to the two low-affinity sites of skeletal fast-twitch TnC in solution; however, Ca^{2+} binding to TnC exhibited positive cooperativity in regulated thin filaments and this was enhanced when S1 was bound to actin. In this regard, both rigor (Bremel and Weber 1972) and cycling (Guth and Potter 1987; Ridgway and Gordon 1984) cross-bridges increase the affinity of TnC for Ca^{2+}. Further experiments in which partial extraction of TnC reduced Ca^{2+} sensitivity of tension (Brandt et al. 1984; Moss et al. 1985), and extraction of whole Tn increased Ca^{2+} sensitivity (Moss et al. 1986a) also provide strong evidence that Ca^{2+} binding to TnC is influenced by the state of activation of other regions of the same thin filament.

The regulatory subunits that mediate cooperativity within the thin filament are not known for certain, although it is likely that Tm is involved (reviewed by El-Saleh et al. 1986). Such cooperativity is a key element in some models of regulation (e.g., Hill et al. 1983) in accounting for Ca^{2+}-dependent variations in tension. Head-to-tail polymerization of Tm within the thin filament (Mak and Smillie 1981) could provide a means for cooperative interactions between functional groups. In a test of this idea Walsh et al. (1984) found that the response of regulated actin-activated myosin ATPase to Ca^{2+} was unaffected by removal of regions of overlap between adjacent Tm molecules. However, Pan et al. (1989) subsequently found that removal of head-to-tail overlap of Tm molecules reduced the cooperativity of S1-ADP binding to reconstitutes thin filaments, and they concluded that overlap of adjacent Tm is necessary for near-neighbor interactions. The difference in results between the two studies may be due to the low ionic strength of the assay system (approximately 20 m*M*) used by Walsh, et al., and their low ratios (1:100) of myosin relative to actin. Thus far, the role of Tm overlap in the apparent cooperativity of tension development has not been studied directly since it has not been feasible to modify the Tm content of skinned fibers. Another protein with possible roles in thin filament cooperativity is TnT, which consists of a globular head (T2) and an extended tail region (T1; Flicker et al. 1982). T1 is long enough to extend to the region of overlap between adjacent Tm molecules (Flicker et al. 1982). Interactions of T1 with the NH_2-terminus of Tm seem to be relatively weak

(Brisson et al. 1986), while interactions with the COOH-terminus are strong. Thus, TnT may influence the interaction of overlapping Tm molecules by its binding to the COOH-terminus of its associated Tm, with little direct interaction with the adjacent Tm.

Fast-twitch and slow-twitch muscles appear to differ with respect to cooperative mechanisms of activation, since tension-pCa relationships are generally steeper in fast-twitch muscles. Such differences may be a manifestation of different Ca^{2+} binding properties mediated by fiber-type specific isoforms of thin filament regulatory proteins (discussed in Sects. 3.3.2 and 3.3.2). On the other hand, the differences may result from fiber-type specific differences in responsiveness to strong binding of myosin cross-bridges to the thin filament. Recent experiments support the second interpretation, in that near stoichiometric substitution of slow TnC into skinned skeletal muscle fibers did not alter the steepness of the tension-pCa relationship (Moss et al. 1991). Further experiments tested the role of strongly bound cross-bridges in cooperatively activating tension (Swartz and Moss 1992). Infusion of *N*-ethylmaleimide S1, a strong-binding derivative of myosin S1 (Williams et al. 1988), increased the Ca^{2+} sensitivity of tension and reduced the slope of the tension-pCa relationship in both fast-twitch and slow-twitch muscles from the rabbit. However, the effects of a given concentration of NEM-S1 were greater in slow-twitch muscle, indicating that slow muscles are more responsive to strongly binding cross-bridges. Thus, as was suspected from the greater steepness of the tension-pCa relationship, fast-twitch muscles are more highly cooperative in the sense that it takes a greater number of strongly bound cross-bridges to cooperatively activate further binding of cross-bridges to the thin filament.

Thus, mechanisms of cooperative activation of tension differ in fast and slow muscles, probably as a consequence of differences in thin filament responsiveness to strongly bound cross-bridges. In addition, some studies have shown that fiber-type specific isoforms of thin filament regulatory proteins also influence Ca^{2+} sensitivity of tension, possibly by affecting Ca^{2+} binding or even the cooperativity of myosin head binding to the thin filament.

3.3.2 Effects of Isoforms of Troponin-T

Experimental evidence from skinned fibers indicates that variable expression of isoforms of TnT results in significant alterations in the Ca^{2+} sensitivity of tension. Schachat et al. (1987) found that natural variations in TnT and Tm isoforms in rabbit fibers were associated with changes

in the tension-pCa relationship. Fibers expressing primarily one of three isoforms of TnT, together with an αα homodimer of Tm, had significantly steeper tension-pCa relationships and greater Ca^{2+} sensitivity of tension than fibers expressing the other two isoforms of TnT together with an α β heterodimer of Tm. These authors interpreted their results as indicating that isoforms of TnT and Tm influence the activation response of thin filaments to Ca^{2+}. Greaser et al. (1988) also related variations in Ca^{2+} sensitivity of tension to variable expression of fast isoforms of TnT in rabbit plantaris fibers. The fibers used for these measurements had approximately equal proportions of α and β Tm and exclusively fast types of TnI and TnC, making it likely that the variation in Ca^{2+} sensitivity of tension was due to differences between fibers in expression of TnT isoforms.

In a study on single skinned fibers from neonatal and adult avian muscles Reiser et al. (1992) found that variations in expression of fast isoforms of Tn-T, identified with an antibody directed to TnT, were strongly related to observed variations in tension-pCa relationships assessed in these fibers (Fig. 12). In chicken ALD muscle, which is slow in the adult, the Ca^{2+} sensitivity of tension was identical at both the neonatal and adult stages of development. Correspondingly, no differences in regulatory protein content were observed on SDS polyacrylamide gels: Tm and the Tn subunits present in these fibers at both

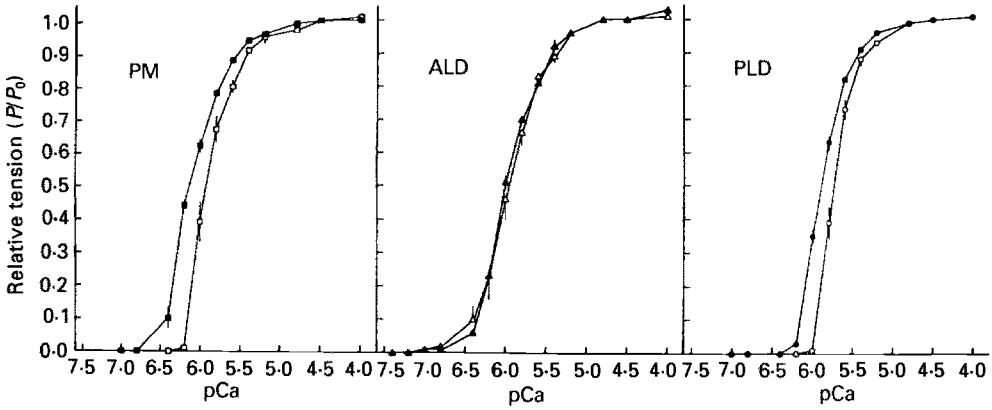

Fig. 12. Tension-Ca relationships obtained from single skinned fibers from chicken pectoralis (PM), anterior latissimus dorsi (ALD), and posterior latissimus dorsi (PLD) at neonatal (*open symbols*) and adult (*closed symbols*) stages of development. The greater Ca^{2+} sensitivity of tension in adult fibers from PM and PLD muscles was attributed to developmental changes in expression of TnT isoforms in these fibers, since isoforms of TnC, TnI, and Tm were identical at the two stages. In ALD there were no differences in Ca^{2+} sensitivity of tension or in regulatory isoform expression at the two stages of development. (From Reiser et al. 1992)

stages of development were the slow isoforms. On the other hand, the Ca^{2+} sensitivities of the adult fast-twitch pectoralis major (PM) and posterior latissimus dorsi (PLD) muscles were different from one another and from the ALD: PM was more sensitive and PLD less sensitive than ALD. For both the PM and PLD muscles, the Ca^{2+} sensitivity of tension was lower at the neonatal stage compared to the adult. Neonatal and adult-stage PM and PLD muscles contained identical fast isoforms of TnC and TnI; however, different isoforms of fast-type TnT were observed between adult PM and PLD muscles and also during the development of these two muscles. Thus, there was a strong relationship between Ca^{2+} sensitivity of tension and the mix of fast TnT isoforms in these muscles.

3.3.3 Effects of Fast and Slow Isoforms of Troponin-C

In both fast-twitch and slow-twitch skeletal muscles, isometric tension increases as a sigmoidal function of pCa, i.e., $-\log[Ca^{2+}]$, but the tension-pCa relationship is less steep in slow-twitch muscle. Such differences are usually interpreted as indicating fundamental differences in cooperativity of tension development, but it is also possible that fiber type specific isoforms of TnC (Wilkinson 1980; Leavis and Kraft 1978) are involved. The best means for testing this idea is to replace endogenous TnC in a single fiber with TnC of the opposite type. Results of these experiments, which are reviewed in Sect. 4.4, do not definitively support the idea that fast and slow isoforms of TnC confer different slopes to the tension-pCa relationships in these muscles.

Ca^{2+} sensitivity of tension is another aspect of regulation that might be expected to differ between fast and slow isoforms of TnC; however, results to date suggest that this is not the case. Stephenson and Williams (1982) found that at a given sarcomere length, the pCa_{50} (i.e., the pCa for half-maximal activation) of tension was greater in slow soleus muscle fibers than in fast extensor digitorum longus muscles of the rat. Similarly, skinned slow-twitch fibers from fish muscles had greater pCa_{50} values than fish fast-twitch fibers (Altringham and Johnston 1982). While some authors have observed greater Ca^{2+} sensitivity of tension in rat fast-twitch than in slow-twitch fibers (e.g., Mounier et al. 1989), Metzger and Moss (1987) observed little difference in Ca^{2+} sensitivity in rat fast-twitch and slow-twitch fibers. Reiser et al. (1992) observed no differences in Ca^{2+} sensitivity of tension in adult avian fast and slow muscles that could be attributed to different isoforms of TnC. Instead, as explained in the previous section, variations in Ca^{2+} sensitivity were

related to variable expression of isoforms of TnT. Since protein isoform composition was not determined in the earlier study by Stephenson and Williams, the difference in Ca^{2+} sensitivity between fast and slow muscles of the rat may be related to variable isoform expression of proteins other than TnC. More detailed work is required to address this point specifically.

3.4 Titin

Titin (also called connectin) is an elastic, sarcomeric protein of approximately 3×10^6 Da (Wang et al. 1979; Maruyama et al. 1981) that spans the gap between the Z-line and the M-line in the sarcomere (Fürst et al. 1988). This protein is thought to account for most of a muscle's resistance to stretch when it is extended under relaxing conditions (Magid and Law 1985). Titin may also play roles in protecting muscle against overstretch (Wang 1991) and in maintaining the central position of the A-band in the sarcomere during force development (Horowits and Podolsky 1987, 1988). In addition, titin may have a role in establishing filament alignment during myofibrillogenesis (Wang et al. 1988; Fürst et al. 1988; Handel et al. 1989,1991; Schultheiss et al. 1990) and could serve as a template to define the length of the thick filaments (Whiting et al. 1989).

New insights into the function of titin have recently been obtained in studies of the stress-strain characteristics of highly stretched skinned fibers from the rabbit (Wang et al. 1991, 1993). A model was proposed in which the gradual increase in resting tension during progressive stretch was ascribed to lengthening of the segment of titin between the Z-line and edge of the A-band. When the elastic limit of this segment of titin is reached, further stretch results in a yield of tension, concomitant extension of part of the A-band segment of titin, and distortion of the ends of the thick filaments. Critical observations in support of this model were obtained using different muscle types. The sarcomere lengths corresponding to the yield points were shortest in adductor magnus and psoas fibers, intermediate in sartorius and longissimus dorsi fibers, and longest in soleus and semitendinosus fibers. SDS polyacrylamide gels of proteins from these muscles revealed three titin species that differed in size, and isoform size was positively related to the mechanical properties. Thus, titin and the myosin-containing thick filament appear to operate as a "dual-stage molecular spring" in that stretch applied to muscle fibers first results in stretch of titin in the I-band region, and once the

strain limit of this part of titin is reached, there is stretch of titin in the A-band region of the sarcomere (Wang et al. 1993).

Several other studies have compared the resting tension characteristics of muscle fibers of different types. Horowits (1992) found that rabbit psoas fibers, which are primarily fast, had greater resting tensions at shorter sarcomere lengths than soleus fibers, which are primarily slow. Titin from the soleus fibers was found to have a slightly higher apparent molecular weight on SDS gels than did the psoas isoform. In humans, the resting tension characteristics of slow-twitch and fast-twitch fibers were identical and were similar to those of rabbit soleus fibers. Somewhat different results have been obtained from fish muscle (Akster et al. 1989), in which red (presumably slow) muscle fibers were less extensible than white (fast) muscle fibers. In this case, the mobility of titin on SDS gels was identical for extracts of red and white fibers obtained from the same muscle.

The electrophoretic migration of titins from embryonic, neonatal, and adult chicken skeletal muscles have been found to differ (Yoshidomi et al. 1985). However, no resting tension measurements have been reported comparing fibers at these developmental stages. Thus, although the hypothesis is that resting tension characteristics of muscle fibers are related to the length of titin, further work is needed to determine whether other proteins or differing stress-strain characteristics of the various isoforms of titin also play a role.

3.5 Parvalbumin

Parvalbumins are low molecular weight, intracellular Ca^{2+} binding proteins that were originally discovered in millimolar concentrations in muscles of lower vertebrates (see reviews by Gerday 1982, Heizmann 1984 and Gillis 1985). Parvalbumins have been proposed to function in speeding the rate of relaxation (Gillis et al. 1982) according to the scheme outlined in Fig. 13. When a muscle is at rest, Ca^{2+} is stored in the sarcoplasmic reticulum, so that the low-affinity, amino-terminal Ca^{2+} binding sites of TnC are unoccupied. Parvalbumin, which contains two metal binding sites (Pechere et al. 1971; Nockholds et al. 1972), would be expected to bind two Mg^{2+} ions under resting conditions. During excitation-contraction coupling Ca^{2+} is released from the sarcoplasmic reticulum and diffuses to the TnC to activate contraction. Parvalbumin would not be expected to compete significantly for this released Ca^{2+}, since the off-rate of Mg^{2+} from parvalbumin is much slower than the binding of

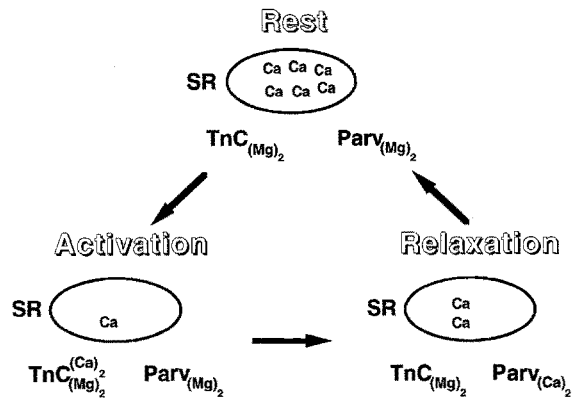

Fig. 13. Proposed role of parvalbumin in muscle relaxation. The figure depicts three states of Ca^{2+} distribution in a muscle cell. When the muscle is at rest, most of the Ca^{2+} is sequestered in the sarcoplasmic reticulum (SR) while TnC and parvalbumin (Parv) both have bound Mg^{2+}. Upon stimulation of the muscle fiber, Ca^{2+} is released from the SR, diffuses through the sarcoplasm and binds to the Ca^{2+}-specific sites on TnC (at the N-terminus; the C-terminal sites contain slowly exchanging Mg^{2+} and bind Ca^{2+} to only a minor extent). The binding of Ca^{2+} by parvalbumin is too slow to compete significantly with the initial activation of the myofibrils. The SR immediately begins to resequester Ca^{2+}, but this process is slower than the exchange of Ca^{2+} for Mg^{2+} on parvalbumin. Thus, parvalbumin is believed to induce relaxation more rapidly than if the SR were acting alone. However, since the SR can successfully compete with parvalbumin for Ca^{2+}, the parvalbumin gradually returns to the Mg^{2+}-bound state once the muscle has relaxed and is again in the resting state. Evidence for this mechanism has been obtained using amphibian and fish muscles in which the parvalbumin content may exceed that of TnC. In mammals, the parvalbumin concentration is usually more than an order of magnitude lower and so would provide little capacity for affecting relaxation rate in the manner outlined here

Ca^{2+} to TnC. However, the affinity of parvalbumin for Ca^{2+} is greater than the affinity of TnC for Ca^{2+}, so that parvalbumin effectively competes for Ca^{2+} bound to TnC. Thus, parvalbumin should contribute to muscle relaxation by binding Ca^{2+}. Of course, the primary mediator of relaxation is reaccumulation of Ca^{2+} by the sarcoplasmic reticulum. Once the muscle is in the resting state, Ca^{2+} bound to parvalbumin would eventually be taken up by the sarcoplasmic reticulum since the Ca^{2+} pump lowers cytoplasmic Ca^{2+} sufficiently to dissociate Ca^{2+} from parvalbumin. Parvalbumin then returns to the state in which two Mg^{2+} ions are bound since myoplasmic concentrations of free Mg^{2+} are in the millimolar range. The adaptive advantage that proceeds from participation of parvalbumin in relaxation is that the binding and reduction of cytoplasmic Ca^{2+} during relaxation is more rapid than that obtained by the sarcoplasmic reticulum alone.

Some aspects of this model of relaxation have been tested in experiments on single frog muscle fibers (Hou et al. 1992a,b). If the model

is correct, stimulating a fiber tetanically should lead to an increase in the time required for relaxation, since Ca^{2+} occupancy of parvalbumin would increase and thus shift Ca^{2+} removal solely to the sarcoplasmic reticulum Ca^{2+} pump. Relaxation time should also correlate with the level of parvalbumin in a fiber. Both of these predictions have been confirmed experimentally (Hou et al. 1992a). Furthermore, since the Q_{10} for Ca^{2+} binding by the sarcoplasmic reticulum is greater than that for Ca^{2+} binding to parvalbumin, the effect of parvalbumin on relaxation time should be greater at 0°C than at 10°C. This has also been found to be the case (Hou et al. 1992b). The presence of parvalbumin therefore appears to be an important adaptation to allow rapid relaxation of muscles that operate under conditions that are suboptimal for Ca^{2+} pumping by the sarcoplasmic reticulum, i.e., in lower vertebrates at low temperatures.

Parvalbumins are also found in birds and mammals, but their physiological function remains controversial. The concentrations of parvalbumin in these animals are typically 20- to 50-fold lower than those found in fish and amphibians. Computer simulations using the concentrations, binding constants, and on/off rates for Ca^{2+} binding to TnC and parvalbumin have led to the conclusion that parvalbumin does not play a significant role in the relaxation process (Robertson et al. 1981). Also, extrapolation of the results of Hou and coworkers (1992b) to 37°C suggests that parvalbumin would play an insignificant role in relaxation in warm-blooded animals. Nonetheless, there is a highly significant correlation between relaxation time and parvalbumin content when individual muscles within a species or the same muscles in different species are compared. The highest parvalbumin content in mammalian muscles has been found in the mouse superficial gastrocnemius, extensor digitorum longus, and tibialis anterior muscles, which have parvalbumin concentrations greater than 4 g/kg and half-relaxation times of less than 10 ms (Heizmann et al. 1982). In contrast, mouse soleus muscle has 0.01 g parvalbumin per kilogram of muscle weight and a half-relaxation time greater than 20 ms. These results agree with studies of fiber type distribution, in which parvalbumin content is high in type IIB fibers, lower in type IIA fibers, and virtually absent from type I fibers (Celio and Heizmann 1982; Schmitt and Pette 1991). However, in man and in horses, muscles with high type IIB fiber content have essentially no parvalbumin (<0.001 g/kg), but this finding is consistent with their longer (>40 ms) times to half-relaxation (Heizmann et al. 1982). Chronic, long- term electrical stimulation of the rabbit fast-twitch tibialis anterior muscle leads to rapid down-regulation of

parvalbumin content, which accompanies the isoform transitions in myosin, Tn, and the sarcoplasmic reticulum (Klug et al. 1983; Leberer et al. 1986; Pette and Düsterhöft 1992; Leeuw and Pette 1993). A potentially useful approach to resolving the physiological role of parvalbumin in avian and mammalian muscles would be to develop transgenic animals having increased expression of parvalbumin.

A novel role for parvalbumin in muscle has been recently proposed by Gailly and coworkers (1993), who found that the *mdx* mouse has tenfold higher parvalbumin mRNA than does normal muscle. In this mouse, the plasma concentration of parvalbumin is much higher than normal, but the muscle content of parvalbumin is nearly normal. A remarkable feature of the *mdx* mouse is that the absence of dystrophin does not lead to the muscle degeneration that is characteristic of some human muscular dystrophies. To account for this difference, Gailly et al. (1993) and coworkers suggested that parvalbumin may act to prevent muscle damage which would otherwise arise due to increases in cytosolic Ca^{2+} which would in turn activate muscle proteases. Conversely, a lack of significant parvalbumin concentrations in human muscles would be expected to result in muscle fiber damage and death.

4 Mechanical Effects Due to Experimental Manipulation of Isoform Content

4.1 Myosin Heavy Chains

The phenotypic expression of MHC isoforms within skeletal muscle fibers is not static but instead is plastic and subject to a number of influences. As discussed in Sect. 3.1, MHC isoform expression varies substantially throughout the course of development (reviewed by Bandman 1985 and by Swynghedauw 1986). In addition, MHC expression in adult skeletal muscle appears to be modulated by factors such as neural activity (reviewed by Pette and Vrbová 1992), hormonal input (reviewed by Mahdavi et al. 1987), and mechanical activity (reviewed by Roy et al. 1991). This plasticity of MHC expression provides a variety of opportunities to perturb expression in order to examine the contractile properties of muscle fibers in relation to MHC expression. Experimental manipulation of isoform content has two distinct advantages compared to observation of naturally occurring variations in isoform content. First, manipulation often results in the expression of

unique combinations of MHC isoforms, as well as unique combinations of MHC and MLC isoforms, that will ultimately provide insight into the relative contributions of these proteins to contractile properties. Second, by altering the time course or the intensity of the experimental manipulation, gradations in the proportions of various protein isoforms are more easily produced. While a number of paradigms have been used to alter the MHC isoform makeup of muscles, we consider here only three experimental manipulations in which measurements of mechanical properties, particularly V_{max}, in both whole muscles and single fibers have been related to induced changes in MHC composition.

4.1.1 Effects of In Vivo Stimulation on MHC Composition and Mechanical Properties of Muscle

The importance of nerve-muscle interactions in the control of muscle phenotype was first recognized in landmark experiments by Buller et al. (1960) in which cross-reinnervation of fast muscles with slow nerves resulted in transformation of contractile properties to slow type. Since that time it has been determined that the stimulation pattern that the muscle receives from the nerve, and not the innervation per se, influences the type of myosin isoforms expressed in the muscle (Salmons and Sreter 1976; Pette and Vrbová, 1985; Hoh and Hughes 1988). Numerous studies have since investigated the sensitivity of the MHC isoform distribution to chronically imposed electrical stimulation paradigms (reviewed by Hoh 1991 and by Pette and Vrbová 1992). These studies have generally shown that muscle can be induced to express slow isoforms of some contractile proteins by exposure to chronic low-frequency (10 Hz) stimulation, while more phasic, higher frequency stimulation protocols result in a slow to fast transformation of some muscles.

Manipulation of MHC isoforms by chronic stimulation would appear to be a nearly ideal method for examining the specific role of the MHC isoforms in determining contractile properties, since the MHC and MLC subunits appear to be replaced with different time-courses during the the stimulation program (Brown et al. 1983, 1985; Seedorf et al. 1983; Leeuw and Pette 1993). However, despite the large body of work examining the effect of chronic stimulation on myosin isoform expression, there is a paucity of studies in which directly measured changes in shortening velocity have been related to shifts in MHC expression in the same fiber. In one study on whole muscles, intermittent high-frequency stimulation of rat soleus muscle in vivo was found to increase the relative expression of the IIx MHC from 3% to as much as 85% of

the total MHC (Schiaffino et al. 1988). This shift in MHC expression was accompanied by an increase in V_{max} to a level intermediate between muscles containing nearly 100% type I MHC and muscles containing predominantly IIb MHC. No measurement of MLC content was made in this study, making it impossible to know the specific contribution of the shift in MHC to the change in V_{max}. Sweeney et al. (1986) examined V_{max} in rabbit tibialis anterior fibers before and after a chronic stimulation protocol that caused a complete transformation of the fibers from IIB to IIA. The V_{max} of the IIA fibers was significantly lower than the IIB fibers. However, this stimulation pattern also changed the MLC content of the fibers, again making it difficult to specifically attribute changes in V_{max} to any particular myosin subunit.

4.1.2 Effects of Altered Thyroid Status on MHC and Mechanical Properties

Hormonal status, particularly levels of thyroid hormone, also has a significant impact on differential expression of MHC isoforms. In fact, it appears that thyroid status predominates over other potential regulators of MHC isoform expression, such as electrical stimulation (Kirschbaum et al. 1990) and mechanical activity (Diffee et al. 1991b; Ianuzzo et al. 1991). Izumo et al. (1986) demonstrated that, in slow muscles, high levels of thyroid hormone are associated with up-regulation of the expression of type II MHC mRNA, whereas chronically low levels of thyroid hormone were associated with up-regulation of type I MHC expression. The site of action of thyroid hormone to modify MHC gene expression has since been described in some detail (Gustafson et al. 1987; Izumo and Mahdavi 1988; Mahdavi et al. 1987).

Two recent studies by Caiozzo et al. (1991, 1992) examined the relationship between V_{max} and changes in myosin isoform expression induced by both hypo- and hyperthyroidism. V_{max} was strongly correlated with the percentage of slow myosin in both the soleus and plantaris muscles of the rat. Manipulation of thyroid status altered the expression of both MHC and MLC isoforms in these muscles, so that changes in V_{max} cannot be specifically related to changes in MHC isoforms alone.

4.1.3 Effects of Activity Patterns on MHC and Mechanical Properties

In addition to responding to imposed patterns of electrical stimulation, expression of MHC isoforms is also sensitive to the degree of chronic mechanical activity imposed on the muscle. An increase in the chronic weight-bearing load on a muscle typically up-regulates the expression

of slow myosin isoforms relative to other isoforms (Tsika et al. 1987b). On the other hand, reducing the weight-bearing load, as in hindlimb suspension, has been shown to increase the percentage of fast-type myosin, especially in muscles that are normally slow (Thomason et al. 1987; Reiser et al. 1987; Diffee et al. 1991a; Fitts et al. 1986). These effects occur despite evidence that the activation patterns of the unweighted muscles are essentially normal (Alford et al. 1987). Studies on whole soleus muscles from the rat have observed an increase in fast myosin content from approximately 5% of total myosin, typical of normal rat soleus, to 15%–20% of the total. Several studies have reported that this unweighting-induced change in myosin content is associated with significantly faster twitch kinetics in soleus muscle (Winiarski et al. 1987; Templeton et al. 1984; Fitts et al. 1986; Diffee et al. 1991a). However, no attempt has been made to relate the degree of change in expression of fast myosin with the degree of change in twitch properties. Such comparisons would in some ways be misleading since there are several factors in addition to myosin isoforms that influence the time course of a single twitch.

Only a few investigators have measured the shortening characteristics of living muscle, such as V_{max}, to investigate the mechanical consequences of changes in myosin isoform content induced by muscle unweighting. Studies on whole muscles showed that the shift toward increased content of fast myosin with a 30% (Diffee et al. 1991a) to 200% (Fitts et al. 1986) increase in V_{max}. Neither of these studies quantified possible relationships between V_{max} and MHC or MLC content, although Fitts et al. (1986) did report that there was no detectable change in the amount of fast MLC isoforms in unweighted muscles relative to controls. Quantification of relationships between V_{max} and MHC content would in any case be difficult in these studies on whole muscle due to heterogeneity of MHC isoform expression among fibers, as well as the fact that not all fibers in the muscle are similarly affected by unweighting (Gardetto et al. 1989; Diffee et al. 1993; McDonald and Fitts 1993).

Single fibers isolated from muscles subjected to unweighting are a more promising preparation for determining possible relationships between contractile properties and MHC content. Reiser et al. (1987) found a 31% increase in mean V_{max} measured in single skinned fibers from unloaded soleus muscles. Those fibers that had higher V_{max} values also contained greater proportions of fast-type MHC. Unweighting-induced changes in MLC isoforms were also observed but were relatively minor compared to the shifts in MHC. Gardetto et al. (1989)

reported that following the unweighting protocol, there were two populations of fibers in the soleus – those with V_{max} values similar to values in control fibers, and those with V_{max} values significantly greater than control. Some fibers in the second group frequently contained both slow and fast isoforms of MHC. A more recent study from the same laboratory yielded some surprising results with respect to MHC composition (McDonald and Fitts 1993). Consistent with earlier results, the percentage of soleus fibers expressing exclusively fast (type IIa) MHC increased from 4% to 29% following 3 weeks of unweighting, and these fibers had elevated myosin ATPase activities and increased V_{max}. However, a significant proportion of fibers exhibited V_{max} and ATPase activities intermediate between control type I and type IIA fibers but had no detectable differences in MHC content from control fibers. At present there is no explanation for this finding, although it is possible that undetected isoforms of slow MHC (Hughes et al. 1993) or isoforms of proteins other than MHC were expressed in these fibers.

4.2 Myosin Light Chain-2

There is now considerable evidence that MLC-2 plays an important role in determining the mechanical properties of fast-twitch skeletal muscle fibers (Moss 1992). For example, partial extraction of LC_2 from rabbit skinned skeletal muscle fibers results in an increase in the Ca^{2+} sensitivity of tension (Hofmann et al. 1990), an increase in the rate of tension development at low levels of Ca2+ (Metzger and Moss 1992), and a decrease in maximum velocity of shortening (Moss et al. 1982, 1983; Hofmann et al. 1990). While the mechanisms underlying the effects of LC_2 removal are not yet known, biochemical experiments showed that removal reduced the actin-activated ATPase of myosins from both heart (Margossian 1985) and skeletal muscles (Margossian et al. 1983), which is consistent with a decrease in mechanical V_{max}. Thus, it is likely that LC_2 modulates the rate of cross-bridge dissociation from actin. Further evidence in support of this idea has been obtained using an in vitro motility assay in which LC_2-deficient myosin moved thin filaments at significantly lower velocities than did LC_2-replete myosin (Lowey et al. 1993).

The dramatic effects on mechanical properties of fast-twitch muscles due to LC_2 removal raises the interesting possibility that various isoforms of LC_2, i.e., slow versus fast in skeletal muscle, confer different mechanical properties to the muscle fibers in which the isoforms are

expressed. While it is evident that fast and slow fibers have distinct mechanical behaviors, the contributions of LC_2 isoforms to these differences are not yet known. Larsson and Moss (1993) observed that human type II fibers expressing both fast and slow isoforms of LC_2 had significantly lower V_{max} values than fibers expressing only the fast isoform. Definitive, quantitative conclusions in this regard will require experiments in which mechanical properties are measured in fast and slow fibers both before and after stoichiometric replacement of the native isoforms of LC_2 with isoforms of the other type.

4.3 Alkali Light Chains

A few earlier studies found strong correlations between V_{max} and alkali light chain composition in rabbit fast-twitch fibers in which MLC composition was thought to be invariant (Sweeney et al. 1986, 1988; Greaser et al. 1988). Additional studies have been done in which attempts were made to experimentally manipulate alkali light chain composition to determine the effects of alkali chains on contraction independent of other factors such as MHC or regulatory protein content. In one such study LC_3 was exchanged for LC_1 in chemically skinned single fibers from rat psoas muscles (Moss et al. 1990). Exchange was effected by bathing the fiber for 60 min at 38°C in a high ionic strength relaxing solution containing 3–5 mg LC_3/ml, and the extent of exchange was quantified by SDS polyacrylamide gel electrophoresis. Fibers bathed in the LC_3 exchange solution exhibited an average increase in V_{max} of 42% and an increase in LC_3 content from approximately 25% of total alkali light chains in the control fibers to 40% following exchange, and a corresponding decrease in LC_1. Thus, from these experiments it is evident that a substantial variation of V_{max} is possibly due to altered proportions of alkali light chain, increasing about 0.4 muscle lengths per second for every 10% increase in the proportion of alkali light chains that are LC_3. Attempts to exchange LC_1 for LC_3 using similar protocols were ineffective in changing either light chain composition or V_{max}.

Lowey et al. (1993) have also studied the possible roles of the alkali light chains and of LC_2 in shortening, but did so by assessing the rate of movement of actin filaments in an in vitro motility assay. In these experiments, sliding velocity was measured in the presence of native myosin, myosin devoid of light chains, and light chain-depleted myosins that were reconstituted with alkali light chains, P-light chain, or both. Velocity was slowest with light chain-deficient myosin, i.e., approximately 9% of control obtained in the presence of native myosin. Addition of

LC_2 to light chain deficient myosin increased V_{max} to approximately 17% of control. Addition of alkali light chains increased V_{max} to about 35% of control, which is consistent with earlier findings that partial extraction of LC_2 significantly reduces V_{max} in skinned fibers (Moss et al. 1982, 1983; Hofmann et al. 1990). Addition of both LC_2 and alkali light chains resulted in virtually complete recovery of velocity to control values. These authors interpreted their results in terms of a model in which the MLCs play a role in transmitting force and movement from thick to thin filaments. Such a model is consistent with the notion advanced by Rayment et al. (1993) that the light chains impart mechanical rigidity to the α-helical portion of myosin S1. At the same time, it is possible that extraction of one or both light chains reduces actin-activated ATPase activity, which is an idea that has not been systematically tested at physiological ionic strength. Most biochemical studies performed at low ionic strength have observed no significant effects on ATPase activity due to light chain removal, although it should be noted that Margossian and colleagues (1983) have reported a 50% reduction in actin-activated ATPase activity in LC_2 deficient myosins from skeletal and cardiac muscles when compared to control.

With regard to possible differential effects of LC_1 and LC_3 on shortening velocity, Lowey et al. (1993) found that sliding velocity was 30% greater with myosin reconstituted with LC_3 as compared to LC_1. This finding agrees qualitatively with previously reported effects of LC_3 exchange into skinned fibers (Moss et al. 1990) and correlations between V_{max} and naturally occurring variations in LC_3 content in rabbit fast-twitch muscle fibers (Sweeney et al. 1986, 1988; Greaser et al. 1988; see Sect. 3.2). While the basis for variations in shortening or sliding velocities with alkali light chain content is presently unknown, it is now evident from a variety of results that light chain isoform content is an important determinant of V_{max}. As suggested by a number of investigators (Sweeney et al. 1988; Moss et al. 1990), it is likely that the range of V_{max}, either fast or slow, is determined primarily by the type(s) of MHC expressed in a fiber present; however, a significant part of the variation of V_{max} within the range of fast velocities can be explained by the relative proportions of alkali light chains that are present. In this regard, Wada and Pette (1993) have shown in both rat and rabbit that there are variations in MLC composition in single muscle fibers having identical MHC composition.

4.4 Troponin-C

Fast-twitch and slow-twitch mammalian muscles have distinct isoforms of TnC that differ in their capacities to bind Ca^{2+} (Wilkinson 1980; Leavis and Kraft 1978). Both isoforms have two high-affinity divalent cation binding sites which have greater affinity for Ca^{2+} than for Mg^{2+} (Potter and Gergely 1975). However, these sites are normally occupied by Mg^{2+} due to the relatively low concentration of Ca^{2+} in resting muscle. Based on their ability to extract TnC from myofibrils using a chelator of divalent cations, i.e., EDTA, as originally described by Cox et al. (1981), Zot and Potter (1982) concluded that occupancy of the high-affinity sites on TnC stabilizes the association of TnC with the remainder of the Tn complex.

Both isoforms of TnC also have low-affinity Ca^{2+}-specific binding sites, which are commonly believed to regulate contraction. Fast-twitch TnC has two low-affinity Ca^{2+} binding sites, while slow-twitch TnC has only one. The significance of this difference between the two isoforms is not completely understood, although it is possible that the isoforms confer different sensitivities to Ca^{2+}. Investigators routinely observe that the slope of tension-pCa relationship in skinned fibers from slow-twitch muscles is less than the slope observed in fast-twitch fibers (e.g., Reiser et al. 1992). The idea that the difference in slopes between fast-twitch and slow-twitch fibers is due to differences in isoforms of TnC has been tested in experiments in which endogenous fast TnC in skinned psoas fibers was nearly stoichiometrically replaced by slow TnC (Moss et al. 1991). The results of these experiments showed clearly that Ca^{2+} sensitivity of tension and the slope of the tension-pCa relationship was unaltered by the exchange. The implication of this finding is that fast and slow isoforms of TnC do not account for differences in the form or midpoint of the tension-pCa relationship in fast and slow muscle fibers. Of course, such an experiment does not eliminate the possibility that the steepness of the relationship is related to interactions of other subunits of Tn (TnT or TnI) with TnC. It should also be noted that earlier similar experiments from the same laboratory (Moss et al. 1986b) observed reductions in the Ca^{2+} sensitivity of tension and slope of the tension-pCa relationship when slow TnC was exchanged into fast-twitch fibers. However, it now seems likely that both effects were due to incomplete reconstitution of those fibers with slow TnC, since TnC deficiency has been shown to reduce the slope of the tension-pCa relationship, thereby shifting the pCa_{50} to higher $[Ca^{2+}]$ (Moss et al. 1985).

5 Relative Effects of Fast and Slow Isoforms of MHC on Shortening Velocity

In appraising the effects of different isoforms of myofibrillar proteins on muscle function it is important to consider that under a variety of conditions muscle fibers coexpress more than one isoform of a given protein. For example, multiple isoforms of MHCs are expressed at various stages of development in both mammalian (Swynghedauw 1986) and avian (Bandman 1985, 1992) skeletal muscles. Coexpression of isoforms of the same protein typically imparts composite behavior to a fiber, which is clearly evident in variations in Ca^{2+} sensitivity of tension when variable ratios of fast isoforms of TnT are expressed (e.g., Schachat et al. 1987) and in variations in V_{max} when variable ratios of fast and slow MHCs are expressed (e.g., Reiser et al. 1985a). The fact that isoforms may be coexpressed in a single muscle fiber leads to questions about the relative effects of two or more isoforms on contractile properties – do these effects add arithmetically, or do slow or fast isoforms have greater influence? This question is best addressed by evaluating relationships between V_{max} and MHC composition, since at present there is insufficient experimental data to consider relationships between mechanical properties and isoforms of other proteins.

In whole muscles, it is now evident that maximum shortening velocity measured by the slack test is determined by the fastest fiber(s) in the muscle, while the maximum velocity estimated by extrapolation from velocities measured at intermediate loads is a function of the force-velocity characteristics of all the fibers that comprise the muscle (Claflin and Faulkner 1985). In single skinned fibers all but a few relationships between V_{max} and MHC content in single skinned fibers described to date have been linear: starting with fibers that have a purely slow isoform of MHC and therefore a slow V_{max}, V_{max} increases in direct proportion to the percentage of faster MHCs coexpressed with the slow isoform (Reiser et al. 1985a, 1987, 1988b), reaching maximum V_{max} in fibers containing only the faster isoform of MHC. This result implies that each isoform contributes equally to measured V_{max}, which is not necessarily the expected result. Rather, one might predict that the relationship between max and the percentage of slow MHC would be curvilinear, with V_{max} decreasing steeply as the percentage of slow MHC increases from zero and decreasing less steeply as the percent approaches 100%. To develop the rationale for this conclusion it is first necessary to recall that force-velocity relationships are curvilinear and can be approximated by an hyperbola over much (Hill 1938) but not the

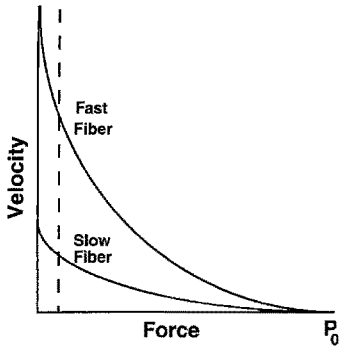

Fig. 14. Idealized force-velocity relationships from fast-twitch and slow-twitch muscle fibers. As shown here, the maximum force per cross-bridge is similar in the two types of muscle, but the extrapolated maximum shortening velocity is substantially less in slow-twitch muscles. dashed line indicates that maximum shortening velocity is substantially reduced in fast-twitch muscles by the imposition of even a small internal load, as would be the case if a fast-twitch muscle expressed a small proportion of slow MHC

entire range of loads between zero and maximal (Edman 1988). As shown in Fig. 14 for a fast muscle fiber, when velocity is plotted against force (or load as percentage of maximum) the relationship is quite steep at loads approaching zero and velocities approaching V_{max}. As discussed above (Sect. 2.3), the primary determinant of V_{max} in muscle is the rate of cross-bridge detachment: cross-bridges that detach slowly after completing their useful working stroke become a load on cross-bridges that are still performing useful work. With this as background it should be possible to predict qualitatively the effects on the force-velocity relationship due to coexpression of MHC isoforms.

A muscle fiber comprised of purely fast MHC has a force-velocity relationship that extrapolates to a fast V_{max} (Fig. 14). If this same fiber now starts to coexpress a small amount of slow MHC, which would be slow to detach during shortening, V_{max} should be reduced as the proportion of slow MHC increases. Due to the steepness of the force-velocity curve near zero load, the addition of an internal load in the form of slowly detaching MHC should cause velocity to decrease rapidly as the percentage of slow MHC increases from zero (Fig. 15).

A muscle fiber comprised of purely slow MHC has a force-velocity relationship that extrapolates to a slower V_{max} (Fig. 14), so that in absolute terms, a slow muscle fiber has a relationship that is less steep near zero load than the relationship for a fast-twitch muscle fiber. Expression of a small amount of fast MHC in an otherwise slow muscle fiber would increase V_{max}, but it is unlikely that the increase would be great. This conclusion is based primarily on the observation that the force-velocity relationship for a fast-twitch muscle is shallow as loads approach maximal. An equivalent model for a slow-twitch fiber with a small amount of fast MHC is one which a population of fast MHC is operating near maximal load on its force-velocity relationship. Thus, increases in fast MHC content and reciprocal reductions in slow MHC

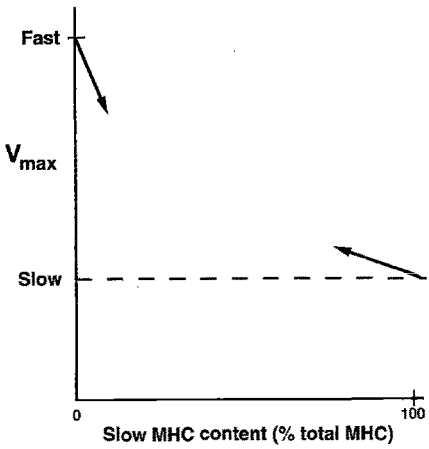

Fig. 15. Likely variations in V_{max} as the percentage of slow MHC expressed in a fiber increases from zero (arrow) or decreases from 100% (arrow). Since total MHC content of a fiber is 100%, increases in slow MHC content would be accompanied by decreases in fast MHC content, and vice versa. Based on the rationale developed in the text, it is unlikely that the relationship between V_{max} and slow MHC content would appear linear the variability in measurement is large with respect to the variation due to curvature in this relationship /or the differences in V_{max} are small between fibers with purely the faster MHC and those with purely the slower MHC

content should result in relatively small increases in shortening velocity (Fig. 15). Therefore, on the basis of the shape of the force-velocity relationship for fast-twitch muscle fibers, it seems that slower isoforms of MHC will have a disproportionately large effect on shortening velocity, reducing V_{max} rapidly as slow MHC content increases from zero, and conversely, acting as an internal load to dampen increases in V_{max} when fast MHC content increases from zero.

From this discussion the relationship between V_{max} and slow MHC content should be biphasic: the expression of small amounts of slow MHC in an otherwise fast muscle fiber would greatly reduce V_{max}, but expression of small amounts of fast MHC in an otherwise slow muscle fiber would only slightly increase V_{max}. In each case the relationships between V_{max} and MHC content could appear linear for modest increases in MHCs of the opposite type; however, when expression of the opposite type of MHC becomes greater, the two relationships must converge, implying that the relationship between V_{max} and MHC composition cannot be linear over its entire range. Of interest in this regard, Maxwell et al. (1982) observed just such a relationship in whole muscles: V_{max} increased at an increasing rate as the proportion of type II fibers increased.

Only a few studies on skinned single fibers have observed curvilinear relationships between V_{max} and the proportion of MHC that are of a slower type (Fig. 9 in Reiser et al. 1988b). In the studies that have reported linear relationships, it is possible that scatter in the data that might arise from variability in determination of either V_{max} or MHC composition may have been too great to discern a curvilinear relationship between these two variables. Determination of relative MHC

content with gel electrophoretic techniques provides an accuracy of at best ±10%, and even this requires careful calibration of staining intensity versus protein mass. Alternatively, curvature of the relationship may be small relative to the observed variability. Nonetheless, the predicted disparity in slopes in the V_{max}-MHC relationship when MHC content is predominantly fast or predominantly slow implies that the curvilinearity of the relationship should be evident when the difference in V_{max} between the two isoforms is great (e.g., Reiser et al. 1985a); however, curvilinearity might not be evident when the difference is small (e.g., Reiser et al. 1988b).

At this point, it is not possible to eliminate the possibility that the relationship between V_{max} and MHC composition in single fibers is actually linear, and it is possible to devise a plausible mechanism for such a case. For example, if the expression of slow MHC in an otherwise fast fiber was restricted to one or a few sarcomeres, V_{max} might be expected to decrease approximately linearly as percent slow MHC increased, thereby increasing the number of sarcomeres in series containing predominantly slow MHC. In this instance, V_{max} would decrease linearly as a function of slow MHC content since overall V_{max} (in μm/s) of a fiber is the arithmetic sum of the V_{max} values of individual sarcomeres.

6 Conclusions

Isoforms of MHCs and MLCs influence the rate of interaction of myosin with actin, while isoforms of thin filament regulatory proteins appear to influence the Ca^{2+} sensitivity of tension. Despite a large body of work to date, relatively little is known about the relative contributions of MHCs and MLCs in determining the rate of cross-bridge interaction assessed by measurements of V_{max}, and little is known about the roles of specific subunits of the regulatory protein Tn and their isoforms in modulating thin filament sensitivity to Ca^{2+} or the cooperative response of the thin filament to strongly bound cross-bridges. These unanswered questions, which are relatively general in nature, point to the need for substantial additional work to characterize the functional consequences of isoform transitions in contractile and regulatory proteins. A recurrent difficulty in studies done to date is that many protein isoforms are coordinately expressed in skeletal muscle, making it difficult to ascribe a particular change in function to a specific change in protein expression. As many as 54 potential combinations of myosin subunits may occur in a single fiber (Staron and Pette 1987). This difficulty points to

the need to isolate the effects of alternate isoforms by performing subunit exchange experiments in skinned single muscle fibers (see Moss 1992 for a review of protein extraction and exchange protocols). Specific subunit exchange allows direct comparison of contractile function without the complication of changes in other protein isoforms. Such exchange protocols are presently feasible for Tn subunits and the light chains of myosin, but not for MHCs or Tm. Although none of the exchange protocols are simple to use, this general approach is likely to yield definitive information about the functional importance of altered expression of myofibrillar protein isoforms. Alternatively, by refining stimulus protocols applied in vivo or by titrating the hormonal status of an animal, it may be possible to limit the effects on gene expression to just one or two proteins. If this were the case, mechanical results obtained from such muscles could clearly be related to specific changes in myofibrillar protein composition.

Virtually no work has yet been done to determine the kinetic transitions in the cross-bridge interaction cycle that are sensitive to altered expression of myofibrillar protein isoforms. This will ultimately be an important area for study to determine molecular mechanisms that underlie mechanical changes that are related to changes in protein isoforms. Investigations such as these could involve classical studies of enzyme kinetics or could take advantage of new methodologies such as flash photolysis of caged compounds to characterize kinetic transitions in intact fibers under physiological conditions. The in vitro motility assay is yet another approach with great potential in this regard, since it may be possible to define the working distance of cross-bridges and the rate of myosin-actin interaction using myosins and thin filaments of known and precisely controlled composition.

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Editor-in-charge: Professor D. PETTE

Hexokinases

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Contents

1 Introduction 66

2 Evolution of Hexokinases 67
2.1 Evolution of Hexose Phosphorylating Activity 67
2.2 Molecular Weights and Product Inhibition of Hexokinases from Various Sources Suggest an Evolutionary Relationship 69

3 Functional Organization of the 100-kDa Mammalian Hexokinases 78
3.1 The Rat Type I Isozyme 79
3.1.1 Association of Catalytic Function with the C-Terminal Half of the Enzyme 80
3.1.2 Association of Regulatory Function with the N-Terminal Half of the Enzyme 82
3.1.3 The Isolated Catalytic Domain Remains Sensitive to Inhibition by Glucose 6-Phosphate: A Revised Evolutionary Scenario 83
3.2 Functional Organization of the Type II and Type III Isozymes 87

4 Structure of Hexokinases 88
4.1 Overview of Structure 88
4.2 Ligand Binding Sites 90
4.2.1 ATP 90
4.2.2 Glucose 98
4.2.3 Glucose 6-Phosphate 100
4.2.4 Inorganic Phosphate 103
4.3 Sulfhydryl Groups and Disulfide Bridges 104

5 Protein Kinase Activity and Phosphorylation of Hexokinases 108

6 Kinetic Mechanism of Hexokinases 111
6.1 Yeast Hexokinase 111
6.2 Mammalian Hexokinases, Types I–III 113
6.3 The Type IV Isozyme of Mammalian Hexokinase 118

7 Mammalian Hexokinase Function in the Physiological Context 120
7.1 Intracellular Distribution 121
7.1.1 Binding of Hexokinase to Mitochondria 122
7.1.2 Binding of Hexokinase to Other Cellular Membranes 138
7.2 Regulation of Mammalian Hexokinase Activity 141

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7.2.1 Short-Term: Regulation of Hexokinase Activity in Response to Transient Changes in Metabolic Status 141
7.2.2 Long-Term: Changes in Hexokinase Activity During Development or in Response to Chronic Alteration in Metabolic Status 142
7.3 Gene Structure and Genetic Disorders 150
7.4 Multiple Forms of the Mammalian Isozymes 152
7.5 The Mammalian Type IV Isozyme 158
7.5.1 Hepatic Glucokinase 158
7.5.2 Pancreatic Glucokinase 164
8 Closing Comments 172
References 174

1 Introduction

The transfer of a phosphoryl moiety from ATP to an acceptor group is an essential step, frequently the initial step, in the metabolism of many molecules. Hence, kinases represent a large and diverse class of enzymes present in organisms across the biological spectrum – microbial, plant, and animal. Although other kinases are mentioned below in a limited fashion, the particular focus in this article is upon kinases that transfer a phosphoryl group from ATP to the 6-hydroxyl of a hexose, most commonly glucose, to yield the 6-phosphoester. In IUB nomenclature these enzymes are referred to as ATP:D-hexose 6-phosphotransferases (EC 2.7.1.1), with the common name of hexokinases. Typically, hexokinases are capable of phosphorylating a range of hexose substrates, but with marked preference for particular hexoses. The IUB also recognizes, as a separate class, ATP:D-glucose 6-phosphotransferase (EC 2.7.1.2), kinases which show high specificity for glucose as a substrate, commonly referred to as glucokinases. The usefulness of this distinction is debatable since even enzymes initially described as glucokinases have been found upon closer examination to be capable of phosphorylating hexoses other than glucose (Maitra 1975; Cárdenas et al. 1984a) and are thus properly included under the EC 2.7.1.1 classification, as emphasized by Cornish-Bowden and Cárdenas (1991) and Iynedjian and Girard (1991). Moreover, as discussed below, extensive similarity in amino acid sequences, deduced from cloned cDNAs, clearly demonstrates that the glucokinases are appropriately included in the hexokinase family, and thus in this review. For the present purposes, we retain the now well-established "glucokinase" designations for these enzymes, even though in the strict IUB sense this designation is incorrect.

Middleton (1990) has recently provided a highly readable and concise review of hexokinases and glucokinases from yeast and mammalian

sources, but to our knowledge this interesting family of enzymes has not been extensively reviewed since the articles by Colowick (1973) and Purich et al. (1973); these may be consulted for discussion of earlier work in the field, which is generally not included in the present treatment. Of more recent vintage is the review by Wilson (1985), which focused on the mammalian hexokinases. In the present article, we undertake to provide an overall view of the relationships between hexokinases (and glucokinases) from a wide range of organisms that have been examined. Despite this attempt to achieve breadth much of the discussion is necessarily focused on mammalian hexokinases and the hexokinases from yeast, since these remain the best characterized representatives of this class of enzymes.

2 Evolution of Hexokinases

Ureta et al. (1987) have presented a thoughtful commentary on evolution of hexokinases that differs in perspective from the comments presented here. The interested reader may wish to consult this for additional information, including an extensive listing of molecular weights of hexokinases from various species; as is seen in the comments below, molecular weight relationships are directly pertinent to speculation about evolution of hexokinases.

2.1 Evolution of Hexose Phosphorylating Activity

Based on comparison of the amino acid sequences of more than 50 "sugar kinases," Bork et al. (1993) concluded that these enzymes could be divided into three distinct nonhomologous families, designated as the hexokinase, ribokinase, and galactokinase families. Within the hexokinase family are kinases preferentially phosphorylating glycerol or various pentoses or hexoses, consistent with the view that this family has maintained structural elements critical for kinase activity while mutations in the substrate binding site generated diversity in substrate preference. Thus, there are members of the hexokinase family that, functionally, are not hexokinases. Conversely, there are functional hexokinases that have distinct evolutionary origins and are not members of the hexokinase family as defined by Bork et al. (1993). In the present discussion, we use the term "hexokinase family" to imply common evolutionary origin, i.e., homologous relationship in the strict biological

sense (Reeck et al. 1987), with attendant similarity in amino acid sequence and structure characteristic of proteins related by divergent evolution.

Based on sequence comparisons, the hexokinase family can be divided into prokaryotic and eukaryotic branches (Bork et al. 1993); the existence of members across this range of organisms indicates an early evolutionary origin for the hexokinase family. No plant hexokinases were included among the sequences compared by Bork et al., but the representation of the hexokinase family in both prokaryotic and eukaryotic organisms makes it seem likely that members of this family will also be found in the plant kingdom when sequences become available (see comments on wheat germ hexokinase below).

The sequence comparisons led Bork et al. (1993) to propose two additional families of kinases capable of phosphorylating various pentose or hexose substrates, namely, the ribokinase and galactokinase families. In addition to those noted by Bork et al., the ketohexokinase (ATP:D-fructose 1-phosphotransferase, EC 2.7.1.3) from rat liver (Donaldson et al. 1993) also appears to be a member of the ribokinase family. The finding of representatives of the ribokinase and galactokinase families, as well as the hexokinase family, in both prokaryotic and eukaryotic organisms makes it likely that the ability to catalyze phosphorylation of sugars arose independently at least three times in early evolutionary history. It is also interesting to note that the galactokinase family includes mevalonate kinase and phosphomevalonate kinase from eukaryotic organisms as well as homoserine kinase from both eukaryotic and prokaryotic organisms; this ability to phosphorylate substrates bearing little if any structural resemblance to carbohydrates again suggests generation of diversity in substrate preference while maintaining a basic "kinase" structure.

ATP (as its Mg^{2+} chelate) is the preferred phosphoryl donor for virtually all sugar kinases that have been examined. Lipmann (1975) suggested that pyrophosphate or polyphosphate may have been used as phosphoryl donors prior to the evolution of ATP-specific kinases characteristic of most present-day organisms. Thus, it is interesting that certain bacterial species, including (presumably) more primitive anaerobes, contain glucose phosphorylating enzymes that utilize polyphosphate as phosphoryl donor (Phillips et al. 1993, and references therein). Phillips et al. (1993) have presented evidence that the glucokinase from *Propionibacterium shermanii* vastly prefers polyphosphate as substrate (by a factor of 10^3 based on k_{cat}/K_m values) but is capable of utilizing ATP to a significant extent. These authors have speculated that the

P. shermanii enzyme might represent an "intermediate" in the evolution of an ancestral polyphosphate-dependent enzyme into the ATP-dependent form characteristic of higher life forms. Since the sequence of the *P. shermanii* enzyme is not known, it is not possible to decide whether it falls into the hexokinase, ribokinase, or galactokinase families defined by Bork et al. (1993). The molecular weight of the *P. shermanii* glucokinase, 30 kDa, suggests that it is not a member of the hexokinase family, but this cannot be totally excluded on this basis (see below).

2.2 Molecular Weights and Product Inhibition of Hexokinases from Various Sources Suggest an Evolutionary Relationship

As discussed in much greater detail below, there are four isozymes of hexokinase found in mammalian tissues (properties nicely summarized by Middleton 1990). Three of these – commonly referred to as types I, II, and III (Katzen and Schimke 1965; Grossbard and Schimke 1966) or less frequently as hexokinase A, B, and C (Gonzalez et al. 1967), respectively – share two properties that are important in the present context, namely, they have molecular weights of approximately 100 kDa and are sensitive to potent feedback inhibition by the product, glucose 6-phosphate. In contrast, a fourth isozyme of mammalian hexokinase, called type IV (Katzen and Schimke 1965; Grossbard and Schimke 1966), hexokinase D (Gonzalez et al. 1967), or, more commonly, glucokinase, has a molecular weight of approximately 50 kDa and is insensitive to inhibition by physiologically relevant concentrations of glucose 6-phosphate (Storer and Cornish-Bowden 1977). In these properties, mammalian glucokinase resembles hexokinases found in yeast. The yeast *Saccharomyces cerevisiae* contains two isozymes, A and B, of hexokinase as well as a third isozyme having a somewhat more marked preference for glucose as a substrate and hence commonly referred to as yeast glucokinase (Colowick 1973; Maitra 1975; Middleton 1990). All three are insensitive to inhibition by physiologically relevant concentrations of glucose 6-phosphate and have a molecular weight of approximately 50 kDa. These observations led Colowick (1973) to postulate an evolutionary relationship, depicted schematically in Fig. 1, in which the 100-kDa mammalian hexokinases evolved by duplication and fusion of a gene encoding an ancestral hexokinase similar to the present-day yeast enzyme. In this view, one of the duplicated catalytic sites (arbitrarily indicated as the site in the N-terminal half of the 100-kDa enzyme in Fig. 1) evolved to take on a regulatory function, with resulting

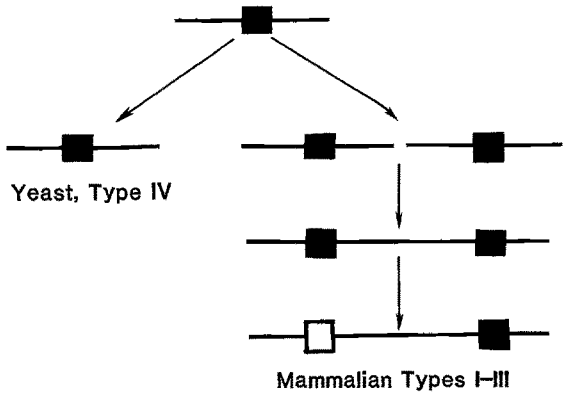

Fig. 1. Evolution of the 100-kDa mammalian hexokinases by gene duplication and fusion, with the allosteric binding site for glucose 6-phosphate arising from a precursor catalytic site (Colowick 1973). *Solid black squares*, catalytic sites of ancestral 50-kDa hexokinase. One branch of the evolutionary pathway leads to the present-day 50-kDa glucose 6-phosphate insensitive hexokinases, such as the yeast or mammalian type IV isozymes. The other branch includes a gene duplication and fusion event to yield a 100-kDa hexokinase, with one of the catalytic sites (arbitrarily, the one in the N-terminal half in this representation) evolving into a glucose 6-phosphate binding site with regulatory function (*open square*). Subsequent gene duplication events would lead to the type I, type II, and type III isozymes found in mammalian tissues

acquisition of sensitivity to glucose 6-phosphate. We show below that this proposed evolutionary scenario requires some modification. However, the suggestion that the 100-kDa hexokinases evolved by a process of duplication and fusion of a gene encoding an ancestral 50-kDa hexokinase related to the contemporary yeast hexokinase has been strongly supported by comparison of amino acid sequences for several hexokinases.

It might be noted here that Blázquez et al. (1993) have recently reported that the isozymes of yeast hexokinase, but not yeast glucokinase, are rather sensitive to inhibition by trehalose 6-phosphate [trehalose being α-glucose (1→1) α-glucose]; intracellular levels of trehalose 6-phosphate were estimated to be in a range that would make this inhibition a significant factor in regulation of hexokinase activity. The inhibition was, however, competitive versus glucose and hence is distinct from the inhibition of the mammalian (and other – see below) hexokinases by glucose 6-phosphate, which is competitive versus ATP.

Among other things, it is evident that the evolutionary scheme (Fig. 1) of Colowick (1973) predicts that there should be internal repetition within the amino acid sequence of the 100-kDa mammalian hexokinases. Moreover, the sequence of the N- and C-terminal halves should be similar not only to each other but to that of the 50-kDa yeast hexo-

kinase. The first direct support for this was provided by Marcus and Ureta (1986), who isolated and sequenced several tryptic peptides from the rat type III isozyme. Close sequence similarities, but not total identities, between some of these peptides clearly suggested their origin from discrete yet related segments within the overall sequence; in terms of the gene duplication-fusion concept, these would be from analogous regions in the N- and C-terminal halves. Moreover, sequences of these and other tryptic peptides derived from the type III isozyme show striking similarity to the sequence of the yeast hexokinase isozymes (Kopetzki et al. 1985; Stachelek et al. 1986). Further direct support for a close relationship between yeast and mammalian hexokinases came from the work of Schirch and Wilson (1987a) in which a reactive glucose analog, N-(bromoacetyl)-D-glucosamine, was used to affinity-label the glucose binding site on rat type I hexokinase. Subsequent tryptic digestion and sequencing of labeled peptides demonstrated close similarity with the amino acid sequence within the proposed glucose binding site of yeast hexokinase (Bennett and Steitz 1980; Harrison 1985).

The cloning of cDNAs for the type I isozymes of human (Nishi et al. 1988) and rat (Schwab and Wilson 1988, 1989) hexokinase permitted comparison of complete sequences. This clearly demonstrated extensive similarity between the N- and C-terminal halves of type I hexokinase, and between these and yeast hexokinase (Fig. 2), consistent with the gene duplication-fusion concept discussed above. Cloning of cDNAs for the rat and human type I isozymes was quickly followed by cloning and sequencing of cDNAs coding for the murine (Arora et al. 1990a) and bovine (Griffin et al. 1991) type I hexokinases, the rat (Thelen and Wilson 1991) and human (Deeb et al. 1993) type II and the rat type III (Schwab and Wilson 1991) isozymes, and the rat (Andreone et al. 1989) and human (Tanizawa et al. 1991; Nishi et al. 1992) type IV hexokinases (glucokinase). The striking sequence similarities between all of these isozymes, between the N- and C-terminal halves of the 100-kDa mammalian enzymes, and between the mammalian hexokinases and yeast (*S. cerevisiae*) hexokinase isozymes A and B (Kopetzki et al. 1985; Stachelek et al. 1986) and glucokinase (Albig and Entian 1988) is evident from the alignments shown in Fig. 2. The gene for a hexokinase from another yeast, *Kluyveromyces lactis*, has also been cloned (Prior et al. 1993); the deduced amino acid sequence (not shown in Fig. 2) shows greater than 70% identity with the *S. cerevisiae* enzymes. Included in Fig. 2 is the deduced sequence of a homologous 50-kDa hexokinase from *Schistosoma mansoni*. We are grateful to Dr. Charles Shoemaker of the Harvard School of Public Health for providing this sequence to

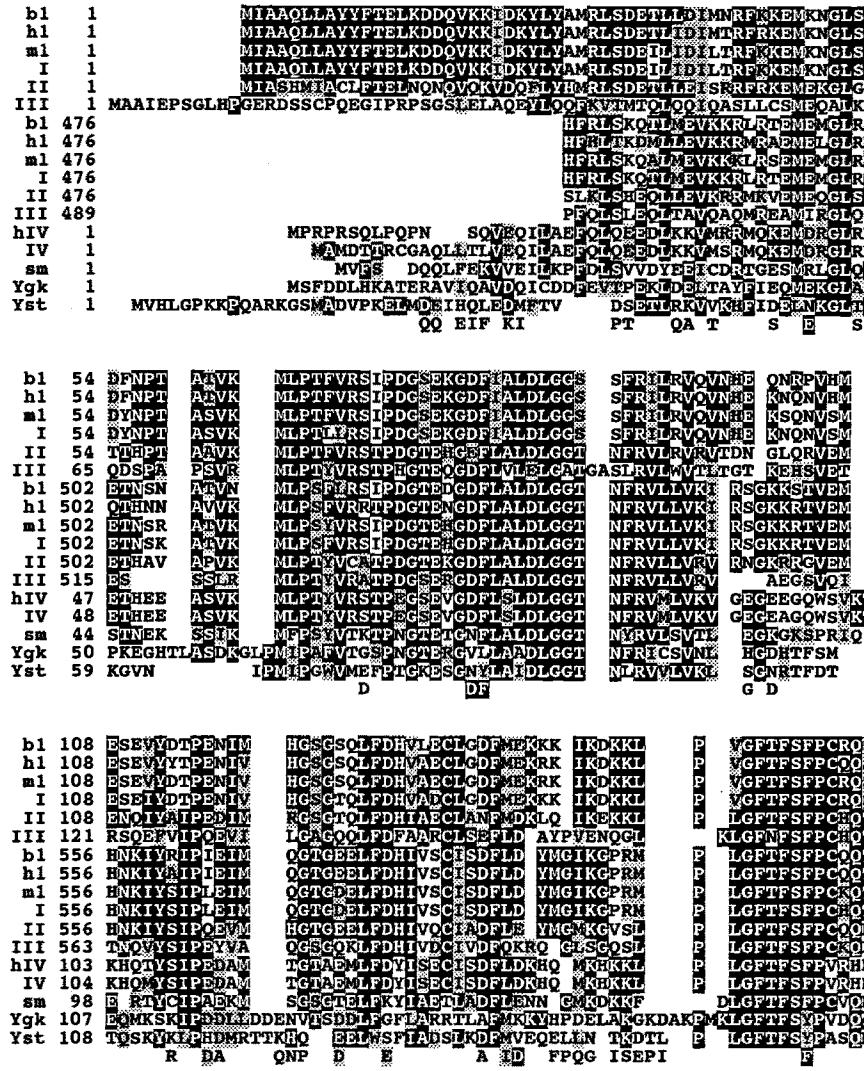

Fig. 2. Comparison of hexokinase and glucokinase amino acid sequences. Sequences compared are: *b1*, bovine type I (Griffin et al. 1991); *h1*, human type I (Nishi et al. 1988); *m1*, murine type I (Arora et al. 1990a); *I*, rat type I (Schwab and Wilson 1988, 1989); *II*, rat type II (Thelen and Wilson 1991); *III*, rat type III (Schwab and Wilson 1991); *hIV*, human type IV (Tanizawa et al. 1991; Nishi et al. 1992); *IV*, rat type IV (Andreone et al. 1989); *sm*, hexokinase from *S. mansoni* (Dr. Charles Shoemaker, personal communication); *ygk*, yeast glucokinase (Albig and Entian 1988); *yst*, yeast hexokinase isozymes A and B (Kopetzki et al. 1985; Stachelek et al. 1986) – the sequence shown is that of the A isozyme, with residues that differ in the B isozyme shown immediately below. Note residue positions indicated at the start of each line; the sequences for the N- and C-terminal halves of the 100-kDa mammalian isozymes have been aligned to show the internal repetition in sequence. *Blackened background*, highlights identity; *stippled background*, conservative changes. (We thank David A. Schwab for preparing this alignment.)

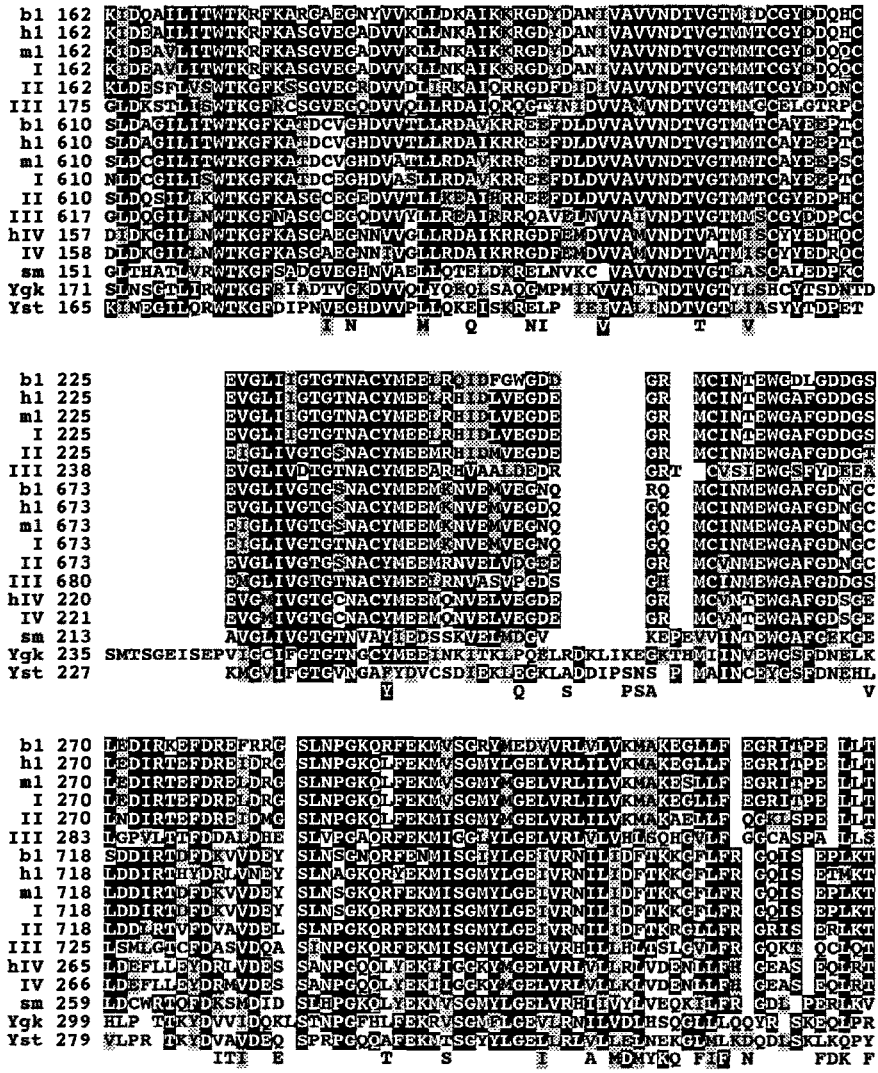

Fig. 2 (*Contd.*)

us prior to publication and for allowing us to include it in the sequence alignment shown in Fig. 2. This is particularly true because the *S. mansoni* enzyme is the first 50-kDa glucose 6-phosphate sensitive hexokinase to be sequenced; the significance of this will become more evident as this discussion develops.

Olafsson et al. (1992) determined the deduced amino acid sequence for a 50-kDa hexokinase from the malarial parasite, *Plasmodium falciparum*. These authors reported 26% sequence identity with the human type I hexokinase (Nishi et al. 1988) and noted conservation of

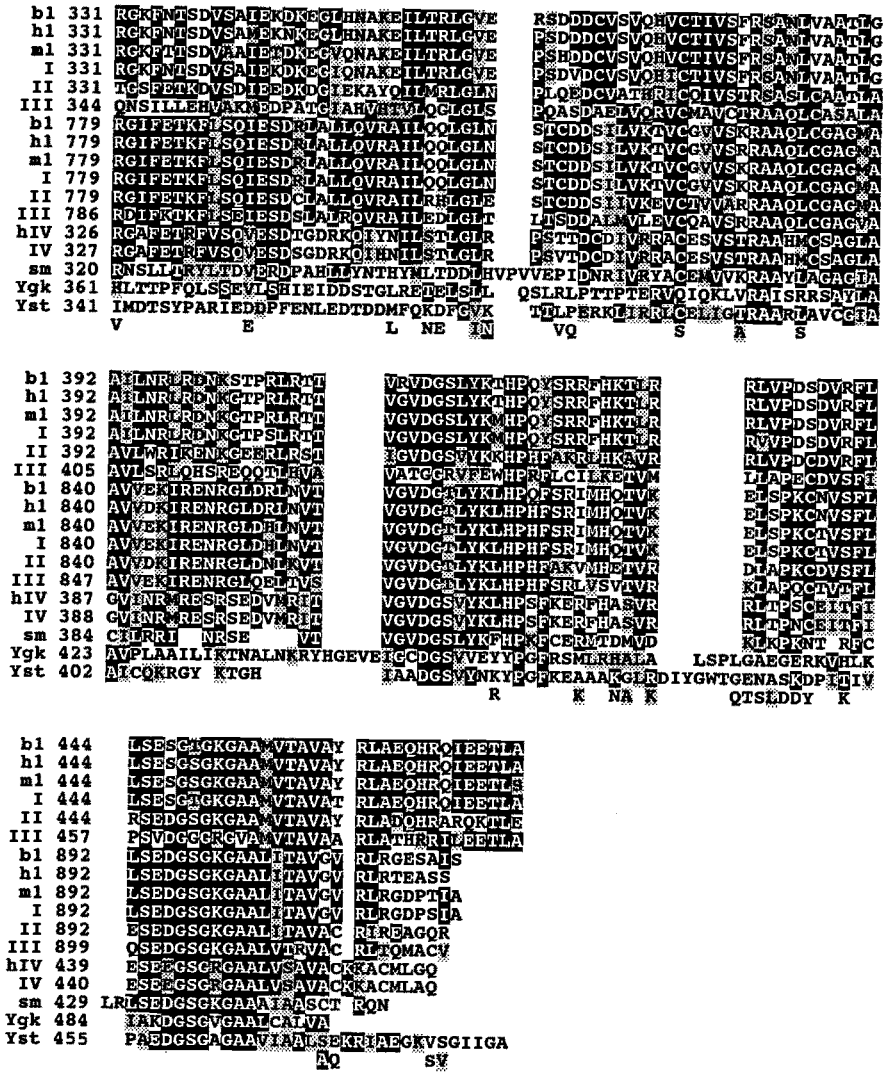

Fig. 2 (*Contd.*)

particular amino acid residues implicated in function, for example, binding of glucose (Bennett and Steitz 1980; Harrison 1985). Barnell et al. (1990) cloned and sequenced the gene encoding a glucokinase from the bacterium *Zymomonas mobilis.* This hexokinase contained only 327 residues, compared with the approximately 500 residues of the *P. falciparum* and yeast enzymes and mammalian glucokinase. Obviously allowing for extensive gaps, Barnell et al. reported approximately 20% identity and 50% similarity when the sequence of the *Z. mobilis* enzyme was compared with those of glucokinases from yeast (Albig and Entian 1988) and rat (Andreone et al. 1989); the *Z. mobilis* enzyme was also included

in the hexokinase family based on the sequence comparisons by Bork et al. (1993). Although Barnell et al. stated that the "glucose-binding domains" found in the yeast and rat glucokinases were absent from the *Z. mobilis* glucokinase, we do not believe this to be the case. Thus, Ser-158, Asp-211, Glu-269, and Glu-302 have been implicated in binding of glucose to yeast hexokinase (Harrison 1985). These residues have been totally conserved in all members of the hexokinase family which have been sequenced (Fig. 2, and Olafsson et al. 1992; Prior et al. 1993). Inspection of the sequence for the *Z. mobilis* glucokinase (Barnell et al. 1990) discloses the presence of residues corresponding to Asp-211, Glu-269, and Glu-302, with adjacent sequence and relative spacing within the overall sequence similar to that seen in the other hexokinases (Fig. 2). Comparisons with the corresponding sequences in yeast hexokinase are:

Z. mobilis	**LIND**FGAHAV	(underlined residue is Asp-102)
S. cerevisiae	**LIND**TVGTLI	(underlined residue is Asp-211)
Z. mobilis	**IET**EGGHID	(underlined residue is Glu-161)
S. cerevisiae	**INC**EYGSFD	(underlined residue is Glu-269)
Z. mobilis	**RFRRVSI**ERIISGPG**LG**	(underlined residue is Glu-191)
S. cerevisiae	**RPGQQAF**EKMTSG**YYLG**	(underlined residue is Glu-302)

Given this conservation of catalytically important residues, it is surprising not to find a Ser, corresponding to Ser-158 in yeast hexokinase, at approximately position 50 in the *Z. mobilis* enzyme. In fact, a sequence of **LGNVS** does appear in exactly this region if the nucleotide sequence is translated in an alternate reading frame (D.A. Schwab, personal communication). This is similar to the corresponding sequence in the yeast enzyme, **LGFTFS**, where the underlined residue is Ser-158; the possibility of a sequencing error must therefore be considered. Focusing on segments thought to be involved in binding of ATP (discussed in detail below), Bork et al. (1993) have also pointed out sequence similarities between the *Z. mobilis* glucokinase and other members of the hexokinase family. Thus, despite its apparently smaller size (which poses intriguing questions about the structure of this enzyme), the *Z. mobilis* enzyme is appropriately considered a bona fide member of the hexokinase family.

The examples discussed thus far make it evident that the members of the hexokinase family (with the apparent exception of the *Z. mobilis* enzyme) fall into either of two classes based on molecular weight, i.e., having molecular weights of approximately 50 or 100 kDa. This is, of

course, as expected based on the gene duplication-fusion evolutionary relationship suggested by Colowick (1973; Fig. 1). Although the sequences are not known, and hence membership in the hexokinase family cannot presently be unequivocally established, it seems likely that at least some other hexokinases with molecular weight of approximately 50 kDa, e.g., from *Drosophila melanogaster* (Moser et al. 1980), brine shrimp (Rees et al. 1989), silkworm (Yanagawa 1978), lobster (Stetten and Goldsmith 1981), starfish (Mochizuki and Hori 1977) and other marine organisms (Mochizuki 1981), and/or the parasitic worm *Angiostrongylus cantonensis* (Oguchi et al. 1979), will also be found to be members of the hexokinase family.

Trypanosomes contain a 50-kDa (Misset and Opperdoes 1984) hexokinase, insensitive to inhibition by glucose 6-phosphate (Nwagwu and Opperdoes 1982; Urbina and Crespo 1984). However, the trypanosomal enzyme reportedly functions as a hexamer (Misset et al. 1986), unlike the yeast and mammalian hexokinases and glucokinases which are monomeric. Is the trypanosomal enzyme a "yeast-type" hexokinase that has, for some reason, evolved to acquire a different functional quaternary structure, or are these unrelated enzymes which coincidentally have a similar molecular weight? We cannot yet answer this question.

The hexokinase from tapeworm *Hymenolepsis diminuta* (Komuniecki and Roberts 1977) has a molecular weight of approximately 100 kDa while the molecular weight reported for the hexokinase from lamprey (Mochizuki 1981) is somewhat smaller, 90 kDa. It is conceivable that these enzymes might be related to the 100-kDa mammalian hexokinases. However, unlike the latter, they do not show the marked sensitivity to inhibition by glucose 6-phosphate. Hence, if they have evolved in a manner similar to that proposed by Colowick (1973; Fig. 1), these enzymes represent an evolutionary branch that has either not acquired or has lost the regulatory characteristics of the 100-kDa mammalian hexokinases. In contrast, the hexokinases from the parasitic roundworm, *Ascaris suum* (Supowit and Harris 1976) and from locust flight muscle (Storey 1980) have a molecular weight of 100 kDa and glucose 6-phosphate sensitivity comparable to that seen with the 100-kDa mammalian enzymes.

Wheat germ presents an interesting situation in which both 50- and 100-kDa hexokinases have been reported to coexist in the same organism (Meunier et al. 1971). Meunier et al. (1971) noted a striking similarity in the amino acid compositions of the 50-kDa wheat germ enzyme and yeast (*S. cerevisiae*) hexokinases, suggesting a possible relationship. Like the yeast enzyme, the 50-kDa wheat germ hexokinase is quite insen-

sitive to inhibition by glucose 6-phosphate (Higgins and Easterby 1974); the sensitivity of the 100-kDa wheat germ enzyme does not appear to have been examined. There is presently no direct evidence for inclusion of either the 50- or 100-kDa wheat germ enzyme in the hexokinase family. However, as mentioned above, it would be surprising if the plant kingdom did not include representatives of this family, and perhaps the wheat germ enzymes will be found to fulfill this expectation.

Hexokinases from other plant sources seem more difficult to fit into the 50-/100-kDa classification. For example, Miernyk and Dennis (1983) reported hexokinase isozymes from developing castor oil seeds to have molecular weights of 38 kDa. While the example provided by the *Z. mobilis* hexokinase (Barnell et al. 1990) makes it evident that bona fide members of the hexokinase family might have molecular weights considerably less than 50 kDa, there is – unlike the situation with *Z. mobilis* hexokinase – presently no basis for inclusion of the castor oil seed enzyme in this family. This is also the case with the hexokinase isozymes from potato tuber (Renz et al. 1993), with reported molecular weights of 66 kDa.

The above discussion makes it evident that many, but not all, hexokinases have molecular weights of either 50 or 100 kDa. Available sequence information (Fig. 2) strongly supports the suggestion that at least some of the 50- and 100-kDa hexokinases are related by a process of gene duplication-fusion (Colowick 1973). A second aspect of the Colowick proposal is that sensitivity to inhibition by glucose 6-phosphate arose by mutation at a duplicated catalytic site (Fig. 1). There are two predictions that follow from this part of the proposed evolutionary scheme: (a) catalytic and regulatory functions should be associated with discrete halves of the 100-kDa hexokinases, and (b) 50-kDa hexokinases should not exhibit the sensitivity to inhibition by glucose 6-phosphate seen with the 100-kDa hexokinases. As is discussed below, the first prediction is fulfilled, at least for the mammalian type I isozyme. However, it is quite clear that sensitivity to glucose 6-phosphate is not restricted to the 100-kDa hexokinases. Thus, the hexokinase from *S. mansoni,* a confirmed member of the hexokinase family with molecular weight of 50 kDa (Fig. 2), exhibits this property (A.G.M. Tielens, personal communication), as does the apparently smaller (Barnell et al. 1990) hexokinase from *Z. mobilis* (Viikari 1988). Other 50-kDa hexokinases, considered possible members of the hexokinase family (see above) and showing reasonably potent inhibition by glucose 6-phosphate, include the enzymes from starfish and other marine organisms (Mochizuki and Hori 1977; Mochizuki 1981; Stetten and Goldsmith

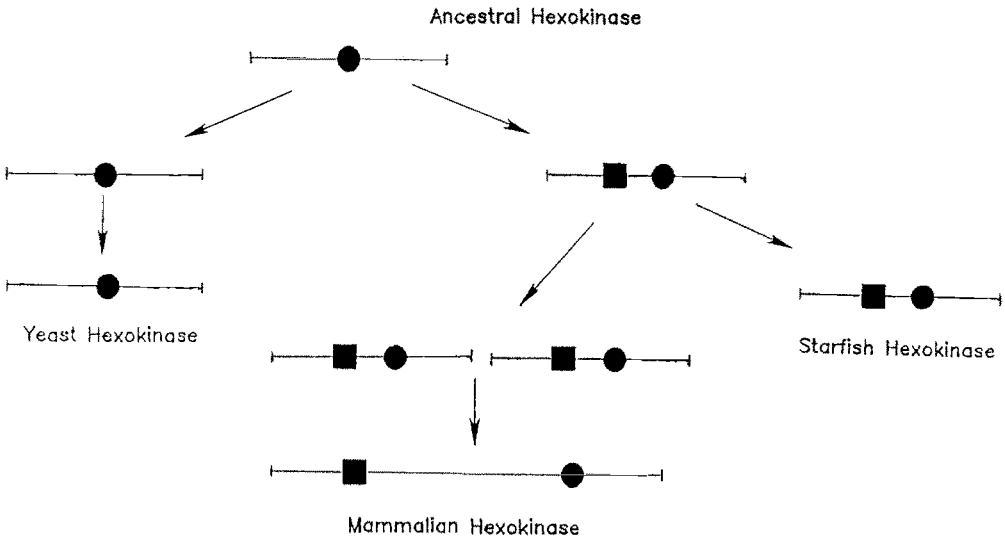

Fig. 3. Evolution of the 100-kDa mammalian hexokinases by gene duplication and fusion, with sensitivity to inhibition by glucose 6-phosphate being acquired prior to the duplication event. *Solid black circles*, catalytic sites of ancestral 50-kDa hexokinase. One branch of the evolutionary pathway led to 50-kDa glucose 6-phosphate insensitive hexokinases, such as found in yeast. In the other branch of the evolutionary pathway, an allosteric regulatory site (*solid black squares*) conferring sensitivity to inhibition by glucose 6-phosphate arose. One evolutionary path from this ancestral form led to the 50-kDa glucose 6-phosphate sensitive hexokinases as found today in starfish and other marine organisms, silkworm, and *S. mansoni*. In the alternative path, gene duplication and fusion led to a 100-kDa glucose 6-phosphate sensitive hexokinase, the prototype for the present-day mammalian isozymes. Subsequent gene duplications would give rise to the mammalian type I, type II, and type III isozymes. Reprinted from White and Wilson (1989) with permission of Academic Press

1981; Rees et al. 1989), and silkworm (Yanagawa 1978). Based on these and other observations, discussed below, a modification of the original Colowick proposal has been suggested (White and Wilson 1989; Fig. 3).

3 Functional Organization of the 100-kDa Mammalian Hexokinases

Colowick (1973) suggested evolution of regulatory function, i.e., allosteric inhibition by glucose 6-phosphate, from a duplicated catalytic site in the 100-kDa hexokinases (Fig. 1). In this section, we review the observations that forced a reevaluation of the proposed evolutionary relationship.

3.1 The Rat Type I Isozyme

The type I isozyme from rat brain was purified in our laboratory many years ago (Chou and Wilson 1972) and has been the focus of much of our work ever since. A modification of the procedure (Wilson 1989), taking advantage of affinity chromatography, has made preparation of the enzyme in reasonable quantity even more straightforward. At the time structural studies on the enzyme were begun the sequence was not known, and the concept that the N- and C-terminal halves of this 100-kDa enzyme might consist of repeated sequence, similar to that of yeast hexokinase, remained speculative (Colowick 1973). This did, however, provide a hypothesis to which experimental findings might be related.

In practical terms, an important advance was elucidation of the cleavage pattern seen with limited tryptic digestion of the native enzyme (Polakis and Wilson 1984). Preferential cleavage occurred at two sites, designated T1 and T2, to yield fragments having molecular weights of 10, 40, and 50 kDa, with partial cleavage intermediates of 60 and 90 kDa. Based on N- and C-terminal sequencing, the disposition of these fragments within the overall sequence was determined to be, in order from N- to C-terminus, 10 kDa, 50 kDa, and 40 kDa, respectively.

Cleavage sites T1 and T2 were subsequently identified (Schwab and Wilson 1989) as Lys-101 and Arg-551 (Fig. 2), respectively. In the absence of denaturing agents the 10-, 40-, and 50-kDa tryptic fragments remained associated with retention of many of the properties of the intact enzyme (Polakis and Wilson 1984). This was interpreted as indicating the existence of strong noncovalent interactions between these fragments within the folded structure of the protein.

Proteolytic cleavage frequently occurs preferentially at relatively unstructured peptide segments linking structural domains within proteins, and structural domains are often correlated with functional domains within proteins (Wilson 1991). Thus the initial interpretation (Polakis and Wilson 1984) of these results was that the 10-, 40-, and 50-kDa fragments correspond to structural, and possibly functional, domains within the hexokinase molecule. This is now considered quite unlikely, with the preferential cleavage at T1 and T2 being related to other structural characteristics of the molecule (Schwab and Wilson 1989); based on the likely similarity between the structures of the N-and C-terminal halves of the type I isozyme and that of yeast hexokinase (see below), T1 and T2 would be located in surface loops expected to be highly susceptible to proteolytic attack.

3.1.1 Association of Catalytic Function with the C-Terminal Half of the Enzyme

The photoactivatable ATP analog, 8-azido-ATP, served as a good substrate for rat type I hexokinase (Nemat-Gorgani and Wilson 1986). Using radiolabeled 8-azido-ATP, this analog was shown to photolabel the enzyme with characteristics expected for true affinity labeling (e.g., labeling was prevented by ATP itself or by competitive inhibitors of ATP binding). The affinity label was associated with the 40-kDa tryptic fragment, indicating that the substrate ATP binding site was located in the C-terminal half of the molecule. Analogous experiments were carried out done with the glucose analog, N-(bromoacetyl)-D-glucosamine, which was also shown to behave as a true affinity label for the glucose binding site on hexokinase (Schirch and Wilson 1987b). Again it was the 40-kDa tryptic fragment that was specifically labeled. Thus, both substrate binding sites, and therefore catalytic function, were associated with the C-terminal half of the molecule.

Independent confirmation of this concept was obtained as a result of finding alternative proteolysis conditions which resulted in cleavage at another site, termed T3 and subsequently identified as Arg-462 (Schwab and Wilson 1989), to yield a 52-kDa fragment corresponding to the N-terminal half of the molecule and a 48-kDa fragment from the C-terminal half (White and Wilson 1987, 1989). Conditions were devised that made it possible to isolate both the 48- and 52-kDa fragments in amounts adequate for characterization. Full catalytic activity was retained in the 48-kDa C-terminal fragment, i.e., the specific activity of the 48-kDa fragment was twice that of the intact 100-kDa enzyme, and no activity was found with the N-terminal fragment (White and Wilson 1989).

Digressing for the moment from our discussion of the type I isozyme, it is interesting to note that Okazaki et al. (1992a) observed that limited tryptic cleavage of the type II isozyme from Ehrlich-Lettre tumor cells generated 40- and 60-kDa fragments. The extensive similarity between sequences of the type I and II isozymes (Fig. 2) portends a corresponding similarity in structure, and hence in regions highly susceptible to proteolytic attack. Thus, although the cleavage site was not defined, it seems likely that the 60- and 40-kDa fragments represent the N- and C-terminal regions, respectively, generated by cleavage of the type II isozyme at a position analogous to T2 in type I hexokinase, i.e., Arg-551; the rat type II isozyme retains an Arg at this position (Fig. 2). In contrast to the type I isozyme, there is no cleavage of the type II hexokinase at a site corresponding to T1 (Lys-101 in type I hexokinase)

and, as noted by Okazaki et al., the type II isozyme contains a Gly at this position (Fig. 2), accounting for the lack of cleavage by trypsin. Okazaki et al. (1992a) reported affinity labeling of the 40-kDa putative C-terminal fragment with the ATP analog, 2′,3′-dialdehyde ATP, results analogous to those obtained with the type I isozyme (Nemat-Gorgani and Wilson 1986).

More recently, assignment of catalytic function to the C-terminal half of rat type I hexokinase has been further supported by results of site-directed mutagenesis experiments (Baijal and Wilson 1992). Every member of the hexokinase whose sequence has been determined possesses a Ser residue at a position corresponding to Ser-158 in yeast hexokinase (Fig. 2); this Ser is involved in hydrogen bonding interactions with the substrate glucose (Harrison 1985). In the 100-kDa mammalian enzymes, this residue is conserved in *both* N- and C-terminal halves. For the rat type I isozyme, these correspond to Ser-155 and Ser-603. Baijal and Wilson (1992) mutated each of these to Ala. Mutation of the serine in the C-terminal half, Ser-603, resulted in drastic reduction of catalytic activity, while the corresponding mutation of Ser-155 in the N-terminal half had no effect on catalytic function. These results are consistent with the view that catalytic function is associated exclusively with the C-terminal half of the enzyme. Why this Ser should have been conserved in a catalytically nonfunctional N-terminal half remains a puzzle.

Arora et al. (1991) also mutated Ser-603 to Ala in the C-terminal half of murine type I hexokinase, reducing the V_{max} to less than 10% of that seen with the wild type enzyme, but with (surprisingly) a reported fivefold *decrease* in K_m for glucose. These investigators also mutated (to Ala) several other C-terminal residues thought to function in binding of glucose (Bennett and Steitz 1980; Harrison 1985); these were Asp-657, Glu-708, and Glu-742, which are analogous to Asp-211, Glu-269, and Glu-302, respectively, in yeast hexokinase (Fig. 2). In each case, the V_{max} was reduced by at least 90%, and the Glu-708 and Glu-742 mutants showed 50- and 14-fold increases, respectively, in the K_m for glucose. The Ser-603Ala, Glu-708Ala, and Glu-742Ala mutations had no significant effect on the K_m for ATP. The activity of the Asp-657Ala mutant was too low to permit evaluation of kinetic parameters. Subsequently, Arora et al. (1992a, 1993) made the analogous mutations in the N-terminal half of the enzyme, with no effect on catalytic activity, in agreement with the findings of Baijal and Wilson (1992) and with the view that catalytic function resides solely in the C-terminal half of type I hexokinase (White and Wilson 1989).

Three groups (Magnani et al. 1992; Chen et al. 1993; Arora et al. 1993) have demonstrated that cDNA coding for the C-terminal half of type I hexokinase can be expressed in *E. coli* with production of a catalytically active "mini"-hexokinase. While further supporting the notion that the C-terminal half can function alone in catalysis, these studies also indicate that folding of the expressed C-terminal half to yield a catalytically active protein can occur in the absence of the N-terminal half of the enzyme.

3.1.2 Association of Regulatory Function with the N-Terminal Half of the Enzyme

In contrast to the ability of hexokinase to phosphorylate a number of hexoses (Sols and Crane 1954), only glucose 6-phosphate and a few closely related analogs serve as potent inhibitors of the enzyme (Crane and Sols 1954; Wilson and Chung 1989). It was in fact this lack of correlation between the ability of various hexoses to serve as substrate and the ability of the corresponding 6-phosphates to serve as inhibitors that prompted Crane and Sols (1954) to make the then-novel proposal of a discrete (from the catalytic site) regulatory site on the enzyme, i.e., an "allosteric" site, a concept developed much more extensively by Monod et al. (1963) several years later.

The extreme specificity of the glucose 6-phosphate binding site thwarted efforts to develop reagents for affinity labeling of the regulatory site, analogous to the approach used for locating the substrate binding sites. An alternative strategy was therefore devised (White and Wilson 1987) based on the observation that binding of ligands frequently stabilizes the ligand binding domain against denaturing agents. In the presence of low (0.6 *M*) concentrations of guanidine hydrochloride, rat type I hexokinase was highly susceptible to tryptic digestion. Inclusion of the substrate analog, N-acetylglucosamine, during the digestion resulted in selective protection of the 48-kDa C-terminal fragment arising by cleavage at T3 (see above); this was consistent with other work, discussed above, demonstrating that the binding site for the hexose substrate was located in the C-terminal region. In contrast, inclusion of glucose 6-phosphate during the digestion resulted in selective protection of the 52-kDa fragment representing the N-terminal half of the enzyme, leading to the conclusion that the regulatory site, to which glucose 6-phosphate bound, was located in the N-terminal half.

The association of catalytic and regulatory functions with discrete halves of the molecule, together with the striking similarity in amino

acid sequence of the two halves (see above), obviously supported the evolutionary scheme (Fig. 1) suggested by Colowick (1973). There was, however, a surprising finding that pointed to an alternative view.

3.1.3 The Isolated Catalytic Domain Remains Sensitive to Inhibition by Glucose 6-Phosphate: A Revised Evolutionary Scenario

The ability to isolate either the N- or C-terminal half, the latter with catalytic activity, permitted characterization of these discrete regions (White and Wilson 1989, 1990). Based on the suggested evolutionary scheme of Colowick (Fig. 1), the catalytic activity of the isolated C-terminal half would not be sensitive to inhibition by glucose 6-phosphate. However, unexpectedly, the activity of the isolated C-terminal domain *was* found to be sensitive to inhibition by the glucose 6-phosphate analog, 1,5-anhydroglucitol 6-phosphate, with a K_i that was indistinguishable from that found for the intact 100-kDa enzyme (White and Wilson 1989). Moreover, the relative ability of other hexose 6-phosphates to inhibit the isolated C-terminal half was quite similar to that seen with the intact enzyme. Clearly, the isolated C-terminal region contained a glucose 6-phosphate binding site with properties similar to the glucose 6-phosphate binding site in the N-terminal half (White and Wilson 1987)!

With the intact 100-kDa enzyme, inhibition by glucose 6-phosphate is antagonized by P_i, an effect attributed to mutually exclusive binding of these two ligands (Ellison et al. 1974, 1975). In contrast, with both the isolated (White and Wilson 1989) and expressed (Magnani et al. 1992; Chen et al. 1993) C-terminal half, P_i itself is an inhibitor. Additional studies (White and Wilson 1989, 1990) disclosed that the N- and C-terminal halves of the enzyme possessed a complete set of binding sites for both substrates, ATP and glucose, for the inhibitory glucose 6-phosphate, and for P_i. Synergistic interactions in the binding of hexoses and hexose 6-phosphates, seen with the intact enzyme (Ellison et al. 1975; Wilson 1979), were also seen with the isolated C-terminal fragment (White and Wilson 1989); this mutual facilitation of binding obviously implies the existence of discrete binding sites for hexoses and hexose 6-phosphates.

It may also be noted here that binding of glucose 6-phosphate to a site having the characteristics (K_i and specificity) associated with the regulatory site and located in the N-terminal half of type I hexokinase (White and Wilson 1987, 1989, 1990) was not dependent on the presence of hexoses, and there was a single glucose 6-phosphate binding site

seen in both the presence and absence of glucose (Ellison et al. 1975; Mehta et al. 1988). This does not support the proposal of Mehta et al. (1988) that formation of the allosteric site is dependent on prior binding of glucose. These authors suggested that, in the absence of glucose, glucose 6-phosphate bound with high affinity to the active site; this is inconsistent with the location of the catalytic site in the C-terminal half (White and Wilson 1989) while, in the absence of glucose, glucose 6-phosphate binds with high affinity to the N-terminal half (White and Wilson 1987). Mehta et al. (1988) also reported positive cooperativity in binding of glucose, which was not seen in similar studies by other workers (Chou and Wilson 1974a; Ellison et al. 1974); it would thus appear that the enzyme preparation(s) used by Mehta et al. had some unusual characteristics.

It was clear from the above observations that this 100-kDa hexokinase was much more complicated than might have been anticipated from the proposed evolutionary scheme of Colowick (1973; Fig. 1). *Sensitivity to inhibition by glucose 6-phosphate was intrinsic to the C-terminal half of the enzyme.* In the latter property as well as other kinetic characteristics, the isolated C-terminal half of type I hexokinase was found to be extremely similar to the 50-kDa hexokinase from starfish (White and Wilson 1989). This led White and Wilson (1989) to the modified evolutionary scheme shown in Fig. 3, in which sensitivity to inhibition by glucose 6-phosphate arose in a 50-kDa hexokinase, similar to the enzymes presently found in *S. mansoni* and in starfish and other marine organisms, *prior to* duplication and fusion of the ancestral gene to yield a 100-kDa hexokinase. Subsequent duplication of this gene would then give rise to the various 100-kDa members of the hexokinase family, the type I, II, and III isozymes.

It is apparent that this modified evolutionary scheme predicts the existence of binding sites for all substrates and effectors in both halves of the 100-kDa hexokinase. This conflicts with studies demonstrating the existence of a *single* binding site for substrate glucose (Chou and Wilson 1974a; Ellison et al. 1974) located in the C-terminal half (Schirch and Wilson 1987b), and a *single* binding site for glucose 6-phosphate (Chou and Wilson 1974a; Ellison et al. 1974, 1975) located in the N-terminal half (White and Wilson 1987). It has been proposed (White and Wilson 1989) that, *in the intact enzyme*, one of each of these sites exists in latent form, presumably as a result of steric obstructions present in the fused molecule. Thus, in the intact enzyme, the N-terminal half retains a latent site for glucose while the C-terminal half has a latent glucose 6-phosphate binding site; when freed from the structural

constraints existing in the intact enzyme, these sites are revealed in the isolated N- and C-terminal fragments. White and Wilson (1990) have presented a schematic representation of the proposed relationship between ligand binding sites in type I hexokinase, integrating this with kinetic studies of the enzyme (Fig. 4); the original reference may be consulted for more extensive discussion of the observations upon which this proposal is based.

Arora et al. (1993) have recently reported expression of both the N- and C-terminal halves of murine type I hexokinase in *E. coli*. Their results confirmed previous work of White and Wilson (1989, 1990) with the N- and C-terminal fragments isolated by limited proteolysis. Again catalytic activity was found to be selectively associated with the C-terminal half (although the specific activity was less than 1% of that seen with isolated enzyme or C-terminal fragment, as used by White and Wilson) and to be virtually identical to the intact enzyme in its sensitivity to inhibition by glucose 6-phosphate (or analogs). Similar results were obtained by Magnani et al. (1992) who expressed the C-terminal half of the human type I isozyme in *E. coli*, finding it to be catalytically active and sensitive to inhibition by glucose 6-phosphate. Arora et al. (1993) also confirmed the previous findings (White and Wilson 1989, 1990) that *both* halves contain sites for binding of ATP and glucose 6-phosphate. Both Magnani et al. (1992) and Arora et al. (1993) suggest that the N-terminal half, and its glucose 6-phosphate binding site, plays some role other than regulation of catalytic activity, and that it is the glucose 6-phosphate binding site in the C-terminal half that serves the regulatory function. Regardless of function, it would seem that this suggestion leads to the expectation that there should be *two* binding sites for glucose 6-phosphate on the intact 100-kDa enzyme, but, as noted above, that is not in accord with observed results (Chou and Wilson 1974a; Ellison et al. 1974, 1975). Neither Magnani et al. (1992) nor Arora et al. (1993) address this aspect of their suggestion. We believe that, despite its complexity, the available results are more reasonably interpreted in terms of a scheme such as that shown in Fig. 4.

How might the type IV isozyme fit into the proposed evolutionary scheme (Fig. 3)? In terms of amino acid sequence, mammalian glucokinase is certainly more closely related to the 100-kDa isozymes than it is to the 50-kDa yeast hexokinases (Andreone et al. 1989; Schwab and Wilson 1991). Yet, like the latter, type IV hexokinase is insensitive to inhibition by glucose 6-phosphate (Storer and Cornish-Bowden 1977). Based on the evolutionary scheme (Fig. 1) proposed by Colowick (1973), Ureta et al. (1987) suggested that type IV hexokinase might have

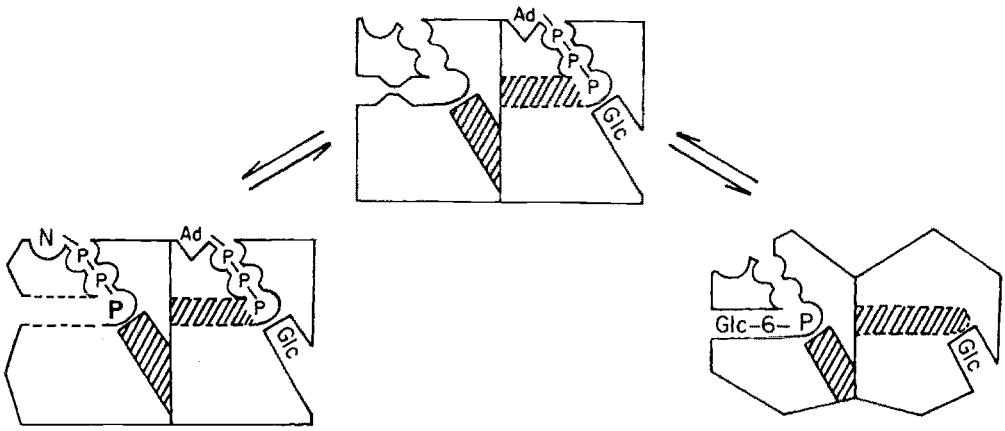

Fig. 4. Schematic representation of binding sites in N- and C-terminal halves of rat type I hexokinase, and their interactions. White and Wilson (1990) should be consulted for a complete presentation of the experimental results leading to formulation of this model. The enzyme is represented as the evolutionary product of duplication and fusion of a gene encoding an ancestral 50-kDa hexokinase that had acquired sensitivity to inhibition by glucose 6-phosphate. The catalytic C-terminal half of the enzyme (*right*) binds glucose and ATP (*center*). Binding of ATP at this substrate site induces a conformational change that precludes binding of glucose 6-phosphate at an allosteric site in the N-terminal half of the enzyme (*left*). Conversely, binding of glucose 6-phosphate at the N-terminal site induces a conformational change that precludes binding of ATP at the substrate site in the C-terminal half (*right*); however, the C-terminal site remains available for binding of glucose and, in fact, binding of glucose and glucose 6-phosphate are synergistic. The N-terminal half also contains a relatively nonspecific binding site for nucleotides (*left*); a nucleoside triphosphate is indicated in this case, with the γ-phosphate (*bold letter*) located in an anion binding site for which the phosphate group of glucose 6-phosphate or P_i can also compete, resulting in mutually exclusive binding of these ligands. In the intact 100-kDa enzyme, the C-terminal half contains a latent (*cross-hatched*) binding site for glucose 6-phosphate and the N-terminal half contains a latent (*cross-hatched*) site for binding of glucose. The latency of these sites may arise from steric factors associated with the fusion of the N- and C-terminal halves. When the two halves are isolated as separate entities, all ligand binding sites in each half are revealed. Reprinted from White and Wilson (1990) with permission of Academic Press

resulted from resplitting of a gene encoding a 100-kDa mammalian-type enzyme, to yield, again, a 50-kDa hexokinase corresponding to the catalytic half of the 100-kDa enzyme; obviously, the resulting 50-kDa enzyme would have lost its sensitivity to glucose 6-phosphate since the regulatory half would no longer be present. Under the modified evolutionary scheme (Fig. 3), resplitting of the gene would *not* automatically result in loss of sensitivity to glucose 6-phosphate; however, this property as well as other changes in kinetic behavior (see below), including markedly decreased affinity for substrate glucose, could have resulted from further mutation after the splitting event. Printz et al.

(1993a) and Kogure et al. (1993) have shown that the intron/exon structure is totally conserved between genomic regions coding for the two halves of rat type II hexokinase, and between these and the gene encoding rat glucokinase (Magnuson et al. 1989). These authors interpreted this as support for the view that the type II isozyme arose by duplication and fusion of a gene encoding an ancestral hexokinase similar to glucokinase. However, it could also be interpreted in support of the proposal that glucokinase arose by resplitting of a gene encoding a 100-kDa hexokinase, with subsequent mutations altering kinetic properties. This modification of the proposals by Ureta et al. (1987), Printz et al. (1993a) and Kogure et al. (1993) has the advantage of explaining the close relationship between the 50- and 100-kDa mammalian hexokinases, deduced from similarities in gene structure as well as amino acid sequence, while preserving the chronological relationship suggested in Fig. 3, i.e., sensitivity to inhibition by glucose 6-phosphate arose *prior to* the gene duplication event giving rise to the 100-kDa hexokinases. Certainly other evolutionary schemes could be envisaged, but further speculation does not seem useful here.

3.2 Functional Organization of the Type II and Type III Isozymes

Given the extensive sequence similarity between the isozymes (Fig. 2), it is apparent that these enzymes must be quite similar in structure. It is perhaps not unreasonable to expect that the functional organization of the type II and type III isozymes might resemble that of the type I isozyme. As noted above, Okazaki et al. (1992a) have presented results indicating labeling of the C-terminal half of type II hexokinase with an ATP analog, consistent with this being a catalytic domain, as found with type I hexokinase. However, this appears to be the only evidence supporting similarity in functional organization of the type I and type II isozymes, and we know of no similar work with the type III isozyme.

Conservation of the functional organization seen with the type I isozyme in the type II and type III isozymes would be the most parsimonious interpretation of the proposed evolutionary scheme (Fig. 3). However, at present it seems prudent to reserve judgement on this matter. As mentioned earlier (and discussed more extensively below), many of the structural features thought to be required for catalytic function have been conserved in both N- and C-terminal halves of all of these isozymes. It is apparent that some difference(s) between the N- and C-terminal halves of the type I isozyme have led to loss of catalytic

function in the N-terminal half. Although some potential candidates have been noted (Schwab and Wilson 1989), the sequence changes responsible for this effect have not yet been identified; it is therefore impossible to determine whether these also occurred in the N-(or C-) terminal half of the other isozymes. It is also possible that sequence changes affecting catalytic activity in N- or C-terminal halves of other isozymes are different from those responsible for loss of activity in the N-terminal half of the type I isozyme. Nor is there any a priori reason to assume that regulatory function is necessarily associated with the N-terminal domain, as seems to be the case for the type I isozyme; the C-terminal domain could conceivably have assumed this function. If functional specialization developed after gene duplication events giving rise to multiple 100-kDa enzymes, this might differ among the isozymes.

4 Structure of Hexokinases

At the present time, the structure of only one member of the hexokinase family has been determined, that of yeast hexokinase (Anderson et al. 1978a,b; Bennett and Steitz 1980; Harrison 1985). However, the extensive similarity in amino acid sequences of the yeast enzyme and other members of this family clearly implies similarity in secondary and tertiary structure (Chothia and Lesk 1986). Thus, the structure of yeast hexokinase has been used as a basis for proposed structures of other hexokinases (Schwab and Wilson 1989; Gidh-Jain et al. 1993). Certainly when the latter structures have been determined directly, they will be found to differ significantly from those of yeast enzyme. Nonetheless, the proposed structures have been shown to be consistent with, and useful for interpretation of, a number of experimental observations (Schwab and Wilson 1989; Smith and Wilson 1991a, 1992; Gidh-Jain et al. 1993; Takeda et al. 1993), supporting the view that the proposed structures are at least reasonable approximations of reality. Thus, although the following discussion is based specifically on the structure of yeast hexokinase, it is reasonable to assume that it will be applicable to other members of the hexokinase family.

4.1 Overview of Structure

Yeast hexokinase consists of two readily distinguishable regions, generally referred to as the "large lobe" and the "small lobe" (Fig. 5). The

Fig. 5. Structure of yeast hexokinase. (We thank Joseph Leykam for providing this "ribbon" representation from coordinates in the Protein Data Bank.)

binding site for glucose (discussed in more detail below) lies at the bottom of the cleft between the lobes. Binding of this substrate induces closure of the cleft (Bennett and Steitz 1980; Steitz et al. 1981; Harrison 1985); the enzyme has been crystallized in both "open" (no glucose) and "closed" (glucose present) conformations (Anderson et al. 1978a,b; Bennett and Steitz 1980; Harrison 1985). A similar bilobar structure and substrate-induced closure of the cleft is seen with other kinases and has been suggested to be a common feature of kinase mechanisms (Anderson et al. 1979).

Both the N- and C-terminal halves of the 100-kDa mammalian enzymes are reasonably presumed to resemble the yeast hexokinase structure. However, comparison of the sequences of the N- and C-terminal halves of the mammalian isozymes with that of yeast hexokinase (Fig. 2) discloses that the C-terminal half of the mammalian enzymes lacks a segment corresponding to approximately 35 residues at the N-terminus of the yeast enzyme. Fusion of two yeast hexokinase molecules, after deleting this segment from the one corresponding to the C-terminal half and manipulation to minimize steric conflicts using computer graphics, led to a proposed structure for a 100-kDa mammalian hexokinase (Schwab and Wilson 1989). Although to a first approximation the

N- and C-terminal halves might be considered equivalent, the discussion above indicates that this is not true in a functional sense. Schwab and Wilson (1989) drew attention to some of the sequence differences (insertions or deletions) that might lead to structural alterations underlying functional differentiation of the N- and C-terminal halves. Based on its greater resistance to proteolytic digestion (Smith and Wilson 1991b), the N-terminal half of rat type I hexokinase was concluded to have a "tighter" structure than the C-terminal half. Differential scanning calorimetry also demonstrated that the two halves differed in their thermal stability, the C-terminal half unfolding before the N-terminal half (White et al. 1990). There is also clear evidence for extensive interactions between the halves, with binding of ligands to either half evoking conformational changes throughout the molecule (Hutny and Wilson 1990; Smith and Wilson 1991b, 1992). Thus, despite their undoubted overall structural similarity, the N- and C-terminal halves should be considered as distinct entities that are functionally coupled within the 100-kDa hexokinase molecule.

4.2 Ligand Binding Sites

4.2.1 ATP

The location of the binding site for substrate ATP and the disposition of the ATP within it have not been demonstrated with certainty. The first proposal came from crystallographic studies of yeast hexokinase by Steitz et al. 1977. It was not possible to crystallize a binary enzyme–ATP complex, but a complex with AMP *was* obtained and the structure determined at 3.5-Å resolution. Assuming that AMP was binding at the same site to which substrate ATP was bound, the proposed location of the ATP binding site was in the large lobe, along the surface bordering the cleft (upper surface of cleft as represented in Fig. 5). Subsequently, the structure of a binary complex of yeast hexokinase with 8-bromo-AMP was determined at 3-Å resolution (Shoham and Steitz 1980); a proposed structure for an ATP-hexokinase complex was derived with the assumption that the location of the adenosine moiety of 8-bromo-AMP correctly represented that of the adenosine portion of ATP, and using the crystal structure of tripolyphosphatetetramine cobalt to model the polyphosphate side chain.

At the time these studies were done the amino acid sequence of yeast hexokinase had not yet been determined, with the result that several of the residues were incorrectly identified in the X-ray structure. To avoid

confusion in this discussion, we have converted residues misidentified in the "X-ray sequence" to their correct identities, as determined from the alignment given by Harrison (1985). Shoham and Steitz (1980) proposed that the adenine ring bound in a "shallow depression" on the surface of the large lobe. Located on either side of, and interacting with, the adenine ring were side chains now identified as corresponding approximately to residues 344–348 and 422–424. The polyphosphate side chain extended along the surface toward the glucose bound at the base of the cleft (details of glucose binding discussed below). This left the γ-phosphate of ATP nearly 6 Å from the position of the 6-hydroxyl of glucose, as determined from the structure of the enzyme-glucose complex. Shoham and Steitz (1980) noted that this was too far to allow the reaction to proceed by the direct phosphoryl transfer mechanism determined for hexokinase. They suggested that a further conformational change, induced by binding of ATP to the enzyme-glucose complex (and hence not seen in the model structure developed for the binary enzyme–ATP complex), must occur.

Flaherty et al. (1990) noted an extraordinary similarity between the structural features of yeast hexokinase and the ATP binding site in the ATPase fragment, obtained by limited proteolysis of the bovine 70-kDa heat shock cognate protein (HSC70). The topology of the ATPase fragment of HSC70 is compared with that of yeast hexokinase in Fig. 6. Both proteins have a bilobar structure, with regions denoted as I and II in the designation of Flaherty et al. (1990) roughly corresponding to the small and large lobes, respectively, of yeast hexokinase. Regions I and II were each subdivided into two domains, designated A and B; the bound ATP in HSC70 was positioned between domains IA and IIA. The resemblance between these domains in the HSC70 ATPase fragment and the corresponding domains in yeast hexokinase is striking (Fig. 6), suggesting that this may correspond to the ATP binding site in hexokinase. A topologically similar ATP-binding motif is found in actin (Flaherty et al. 1991), with ATP binding in a manner equivalent to that seen with HSC70 (Flaherty et al. 1990).

Bork et al. (1992) superimposed the "structural cores" of actin, HSC70, and hexokinase; these were comprised of substantial portions (approximately 45% of the residues) of the ATP-binding domains, IA and IIA (as defined above). Using a "pattern recognition" approach, in which amino acid residues are defined by the properties of their side chains (e.g., hydrophobicity, size, charge, etc.) rather than their identity per se, Bork et al. (1992) discerned five discrete sequence motifs that were characteristic of the ATP binding sites (either demonstrable or

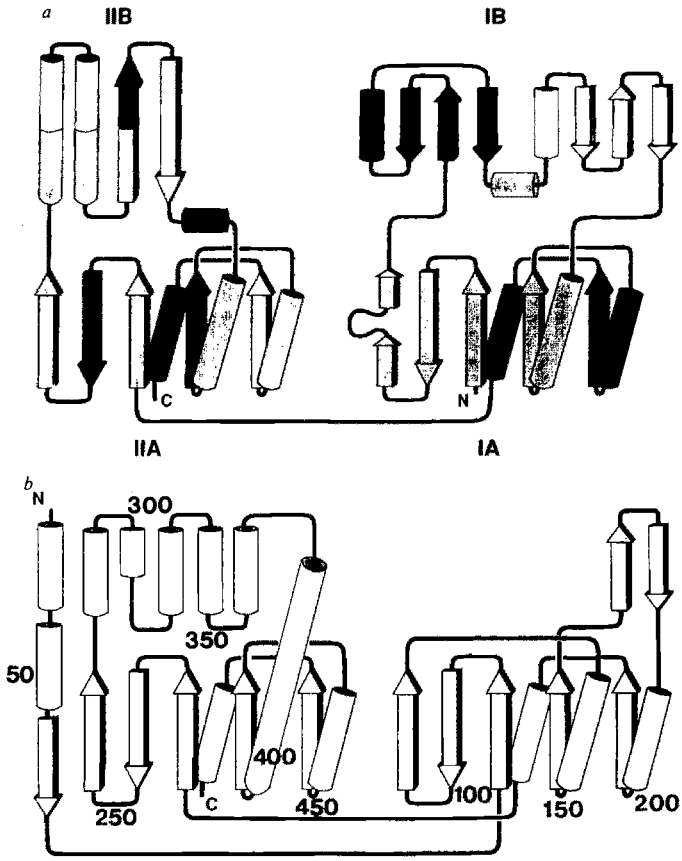

Fig. 6. Topological comparison of the ATPase fragment of bovine 70-kDa heat shock cognate (HSC70) protein and yeast hexokinase. *Above,* ATPase fragment; *below,* yeast hexokinase. *Cylinders,* **a**-helices; *arrows,* **b**-strands. The overall structures are divided into two regions (designated *I* and *II*), with each of these being further divided into two domains (*A* and *B*). The striking similarity in the topology of domains IA and IIA of HSC70 and the corresponding domains in yeast hexokinase is readily apparent; residues within these domains are involved in binding of ATP. In contrast, the IB and IIB domains show no similarity. To facilitate location of sequences discussed in the text, the *approximate* position of residues within the hexokinase sequence are numbered at intervals of 50. Reprinted with permission from *Nature* (Flaherty et al. 1990). Copyright 1990 Macmillan Magazines Limited

putative) in these and other ATP binding proteins. These were referred to as PHOSPHATE 1, PHOSPHATE 2, CONNECT 1, CONNECT 2, and ADENOSINE based on the nature of their function. The consensus sequences and disposition of these regions within the structure are shown in Fig. 7. PHOSPHATE 1 and PHOSPHATE 2 are regions interacting with the phosphate side chain while ADENOSINE is a segment interacting with the adenosine moiety; in hexokinase, ADENOSINE

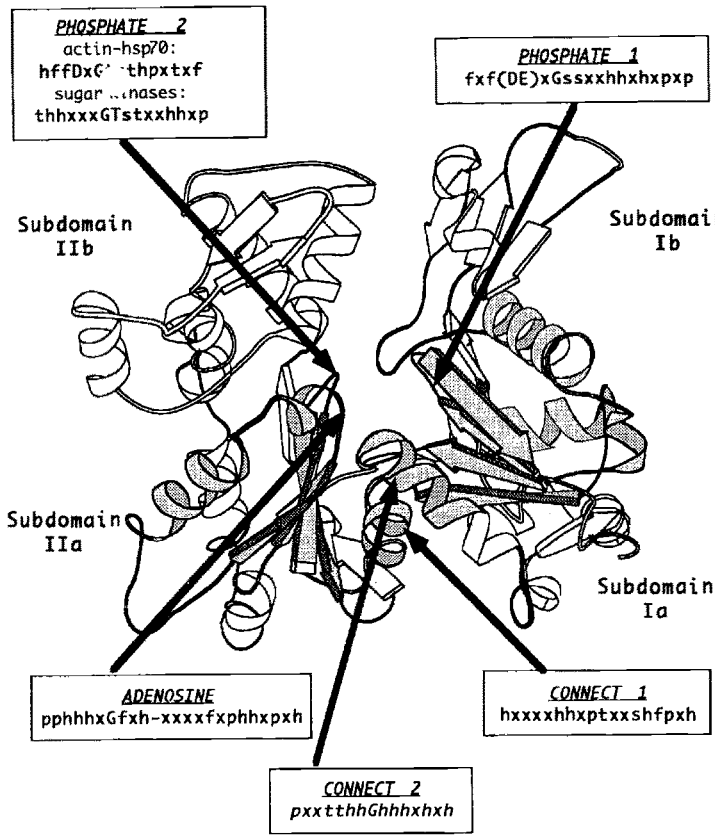

Fig. 7. Actin structure and location of regions involved in binding of ATP. The overall structure can be divided into domains IA, IB, IIA, and IIB, analogous to the structural divisions in HSC70 and yeast hexokinase shown in Fig. 6. The ATP binding site is located between domains IA amd IIA. Approximate location and consensus sequences of segments involved in binding of ATP are indicated. Reprinted from Bork et al. (1992) with permission of Dr. Peer Bork

corresponds to residues 411–439 (Fig. 8), and thus includes one of the segments, residues 422–424, identified by Shoham and Steitz (1980) as being in proximity to the adenine ring. CONNECT 1 and CONNECT 2 are helical regions linking domains IA and IIA; these are in contact and suggested to serve as a hinge upon which interdomain movement occurs.

The amino acid sequences in these five regions are shown in Fig. 8. It is evident that absolute conservation of identity is required at a rather limited number of positions; thus, detection of the similarity between the corresponding regions in these and other proteins was greatly enhanced by – indeed, probably only possible with – the "pattern recognition" approach. Based on detection of corresponding segments

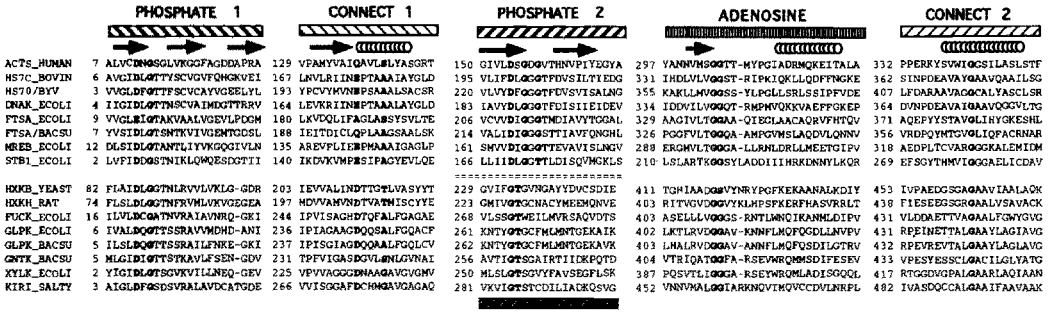

Fig. 8. Alignment of sequences in the ATP binding motif identified by Bork et al. (1992). The sequences correspond to regions indicated in Fig. 7 and were identified by Bork et al. (1992) after analysis of the sequences of a number of ATP binding proteins from a variety of eukaryotic and prokaryotic sources. These include yeast hexokinase (HXKB_YEAST) and rat type IV hexokinase (HXKH_RAT); see Bork et al. (1992) for identification of other proteins. Reprinted from Bork et al. (1992) with permission of Dr. Peer Bork

at analogous positions within the sequence of several other proteins, Bork et al. (1992) predicted that these proteins, whose actual structure was then unknown, would be found to possess similar structural regions involved in nucleotide binding; subsequent determination of the structure of glycerol kinase (Hurley et al. 1993) revealed the predicted structural motif, which is reasonably presumed to serve in binding of ATP.

Although similar in the other four segments, two distinct subfamilies of proteins were recognized based on sequence characteristics of the PHOSPHATE 2 region (Figs. 7, 8). Interestingly, one was primarily populated by various kinases, while the other consisted of proteins utilizing ATP for purposes other than as phosphoryl donor. The distinguishing feature of the kinases was a highly conserved GT in PHOSPHATE 2. This is found in all known members of the hexokinase family (Bork et al. 1993, and Fig. 2) *with the exception* of the N-terminal half of type III hexokinase (Fig. 2) which contains an Asp at this position; since the PHOSPHATE 2 segment is involved in interactions with the negatively charged phosphate side chain, this substitution should certainly adversely affect, and probably preclude, nucleotide binding to the N-terminal domain of type III hexokinase.

Together, domains IA and IIA largely comprise the ATP binding region in these proteins. However, the ADENOSINE segment (located in domain IIA) forms only one side of the adenine binding pocket. In both actin (Flaherty et al. 1991) and HSC70 (Flaherty et al. 1990) another side is formed by residues found in a helical segment from domain IIB (residues 210–214 in actin, and residues 268–272 in HSC70). It is interesting to note that a helix is found in comparable position in

yeast hexokinase (Harrison 1985), and includes residues 344–348, also identified by Shoham and Steitz (1980) as being in proximity to the adenine ring. One might anticipate finding a comparable structure in other proteins utilizing this ATP binding motif. There does not appear to be significant sequence similarity in this region, however, and these segments were not identified in the profile analysis of Bork et al. (1992).

Noting the overall structural symmetry in this region, as well as sequence similarity between the symmetrically placed phosphate binding loops, Bork et al. (1992) suggested that this ATP binding site had arisen by duplication and fusion of an ancestral gene coding for the equivalent of the IA (or IIA) domain. They postulated that noncovalent association of this ancestral protein resulted in formation of a homodimer possessing ATP binding ability. Gene duplication and fusion would yield a monomeric ATP binding protein equivalent to domains IA plus IIA; subsequent acquisition of domains, corresponding to IB and IIB and conferring other capabilities, would lead to a functionally diverse family of proteins sharing a common ATP binding motif. Mutations in the PHOSPHATE 2 region gave rise to one branch of this family leading to the hexokinases.

It should be noted that well before the recent work of Bork et al., others had proposed an evolutionary relationship between the two lobes of yeast hexokinase. Thus, based on structural comparisons, Rossmann and Argos (1977) proposed "divergence of the two hexokinase domains from a common ancestor," although Harrison (1985) has challenged that view. McLachlan (1979) also proposed, again purely on structural grounds, that the central core of yeast hexokinase had evolved by gene duplication and fusion, with subsequent acquisition of peripheral structural components having functional significance.

It is evident from the above comments that the location of the ATP binding site proposed by the Steitz group (Steitz et al. 1977; Shoham and Steitz 1980) is generally consistent with the location deduced by comparison with the ATP binding sites of the (presumably) homologous proteins, HSC70 (Flaherty et al. 1990) and actin (Flaherty et al. 1991). The placement of the adenine moiety is well within the large lobe. However, other work has emphasized the small lobe as the site of ATP binding. Arora et al. (1990b) synthesized a 50-amino acid peptide having the sequence of residues 78–127 in yeast hexokinase. This roughly corresponds to the first three β-strands in domain IA (Fig. 6). Included in this region is Lys-111, which was labeled by a reactive analog of ATP, pyridoxal 5-diphospho-5′-adenosine, and suggested to be involved in binding of the nucleotide (Tamura et al. 1988); specifically, electrostatic

interactions of the Lys residue with the phosphate side chain of ATP were proposed. Arora et al. (1990b) showed that a fluorescent ATP analog, 2′,(3′)-*O*-(2,4,6-trinitrophenyl)-adenosine-5′-triphosphate (TNP-ATP), bound with nearly 1:1 stoichiometry to the peptide, and that binding was prevented in the presence of ATP itself. Glucose had no effect on binding of TNP-ATP, not surprisingly since residues involved in binding of glucose were not included within the peptide sequence. In contrast, binding of TNP-ATP to yeast hexokinase was facilitated in the presence of glucose, as previously seen with binding of ATP itself (Peters and Neet 1978; Steitz et al. 1981).

Whether the binding of TNP-ATP (and ATP) to this peptide is fortuitous or indeed models interaction of nucleotides with the intact enzyme remains unclear. This peptide does not include the ADENOSINE sequence (Fig. 8) identified by Bork et al. (1992) as being involved in interactions with the adenosine portion of ATP, nor does it include residues 344–348, proposed by Shoham and Steitz (1980) to be interacting with the adenine ring. Despite this, it is likely that interaction of TNP-ATP with the peptide occurs primarily, and probably exclusively, through the adenine and/or ribose moieties. Binding of TNP-ATP to the peptide was not affected by the presence or absence of Mg^{2+}. Although not discussed by Arora et al. (1990b), it seems reasonable to interpret the latter observation as indicating that, despite the presence of a Lys residue analogous to Lys-111 in the intact yeast enzyme, the peptide does not interact with the phosphate side chain of TNP-ATP (or ATP itself); such interactions are expected to be sensitive to chelation status of the side chain. Mutation of the corresponding Lys residue in type I hexokinase, Lys-558, had a rather modest effect on V_{max} and *no* effect on the K_m for ATP (Arora et al. 1991). Moreover, although the N-terminal half of type I hexokinase retains the ability to bind ATP in manner sensitive to chelation status of the phosphate side chain (White and Wilson 1990), there is no Lys (or Arg) at a position analogous to Lys-111 of yeast hexokinase. Together with other considerations (Schwab and Wilson 1991), these observations argue against a role for Lys-111 in interactions with the phosphate side chain as proposed by Tamura et al. (1988).

Based on characteristic amino acid sequence features seen with other nucleotide binding enzymes, Schwab and Wilson (1988) proposed a location for the ATP binding site. A hydrophobic surface formed by the β-sheet structure (residues 81–89, 92–99, 100–114, 153–158, and 206–210) within the small lobe (domain IA in Fig. 6) was suggested to interact with the adenine ring. With the exception of residues 100–114,

which are near the surface where greater variation in side chains might be tolerated without effect on structure, these sequences are highly conserved among the hexokinases (Fig. 2). The results of Arora et al. (1990b) are consistent with this suggestion since their ATP binding peptide included this region. Although placement of the adenine ring in the small lobe contrasts with the location suggested by the work of Steitz and his coworkers (Steitz et al. 1977; Shoham and Steitz 1980) as well as the analysis of Bork et al. (1992), other interactions suggested by Schwab and Wilson (1988) could be accommodated within the proposal of Bork et al. (1992). For example, Schwab and Wilson drew attention to a sequence Gly-X-Gly-X-X-Gly(Ala) found in several nucleotide binding enzymes. Located at the (positive) N-terminus of a helix dipole, interactions with the negatively charged phosphate side chain may occur. This structural feature is found at positions 459–464 in yeast hexokinase, and the sequence is highly conserved within the hexokinase family (Fig. 2). Interactions of the phosphate side chain with this helix dipole may supplement interactions with the PHOSPHATE 2 region defined by Bork et al. (1992), both being adjacent within the structure. Site-directed mutagenesis studies aimed at evaluation of the roles of these and other residues suggested to be involved in nucleotide binding are presently underway in our and probably other laboratories.

It is quite possible that the seemingly conflicting proposals described above may not, in fact, be in conflict. Shoham and Steitz (1980) pointed out that, in the "closed" form of yeast hexokinase, induced by binding of glucose, there would be additional contacts between the bound ATP and residues in the small lobe. It is not unreasonable, nor inconsistent with the structures determined by Shoham and Steitz (1980), to suggest that closing of the cleft brings the β-sheet region of the small lobe into position to interact with the adenine moiety of ATP. Indeed, Shoham and Steitz (1980) suggested that glucose-induced interaction of the small lobe (in addition to the large lobe) with bound ATP may be a requirement for proper ordering of the polyphosphate side chain, required for phosphoryl transfer, and may also be responsible for increased affinity for ATP seen in the presence of glucose (DelaFuente and Sols 1970; DelaFuente et al. 1970; Roustan et al. 1974; Peters and Neet 1978; Viola et al. 1982; Woolfitt et al. 1988).

There are several diastereoisomers of the ATP-Mg^{2+} chelate. Both the yeast (Jaffe and Cohn 1979) and mammalian (Darby and Trayer 1983) hexokinases have been found to be identical in their stereospecificity, providing further evidence for the conserved nature of the ATP binding site in the hexokinase family.

Olsen and Reed (1993) have recently deduced the structure of a Mn^{2+}-ADP-nitrate-lyxose complex at the active site of yeast hexokinase; this could be considered analogous to the ternary complex of hexokinase with Mg^{2+}-ATP and glucose. According to Olsen and Reed (1993), no residues from the enzyme interact with the divalent cation. In contrast, Shoham and Steitz (1980) detected interaction between the cation and the side chain of Thr-234 (misidentified as Ser-212 in the "X-ray sequence") in the modeled structure for the binary complex of yeast hexokinase with ATP-Mg^{2+}. It should be noted that Thr-234 and bracketing Gly residues are part of the PHOSPHATE2 segment (Bork et al. 1992), and are highly conserved among the hexokinases (Fig. 2), consistent with functional importance. This difference between the findings of Olson and Reed (1993) and of Shoham and Steitz (1980) may be a reflection of structural differences between the binary and ternary complexes, as deduced by Shoham and Steitz (1980) and discussed above.

4.2.2 Glucose

In contrast to the situation with ATP, the glucose binding site can be located with greater confidence since the complex of glucose with the yeast enzyme has been crystallized (Bennett and Steitz 1980; Harrison 1985). As noted above, the X-ray structural studies were carried out prior to determination of the amino acid sequence, leading to some uncertainty or incorrect identification of specific residues. As a result, reference to some of the earlier studies may be confusing. In the present discussion we rely heavily on the most recent work (Harrison 1985), with identification of residues as determined from the amino acid sequences deduced from cloned genes for the yeast hexokinases (Kopetzki et al. 1985; Stachelak et al. 1986). Corresponding residues in other hexokinases can be ascertained by reference to Fig. 2.

A conformational change induced by binding of glucose (or other hexose substrates) is critical for catalysis by yeast hexokinase (Steitz et al. 1981) and type I (and presumably other) mammalian hexokinase (Wilson 1978a, 1979); interactions with the 6-hydroxyl group are essential for this conformational change (Shoham and Steitz 1982). The latter is a consequence of, not preliminary to, binding of the hexose. Thus, Harrison (1985) divides the binding process into two phases, an initial "lock and key" recognition by an "open" conformation, followed by closing of the cleft to yield a conformation in which the mode of interaction between enzyme and substrate has been altered. Residues thought to be involved in hydrogen bonding interactions with the hydroxyls of

Table 1. Hydrogen bonding contacts between glucose and yeast hexokinase in open and closed conformations (adapted from Harrison 1985)

	Interacting residues in yeast hexokinase[a]	
Hydroxyl position	Open conformation	Closed conformation
6	Gly-235	Asp-211
5	None	None
4	Asn-210	Asp-211
	Asp-211	
	Asn-237	
3	Asn-210	Ser-158[b]
	Glu-269	Glu-269
2	Glu-269	Glu-302
1	Val-236	Glu-302
	Glu-302	

[a]Interactions are with carbonyl oxygens involved in peptide bonds of Ser-158, Gly-235, and Val-236. Interactions are with side-chain atoms in other residues.

[b]Harrison (1985) identifies the hydrogen bonding to be through the carbonyl oxygen of Ser-158. However, this seems questionable since mutation of Ser-158 to Ala has a marked adverse effect on catalysis (Arora et al. 1991; Baijal and Wilson 1992), implying functional significance for hydrogen bonding through the side-chain. A Ser residue is also conserved at this position in every hexokinase sequence that has been determined (Fig. 2); it is difficult to imagine why this should be the case if interactions via the side-chain were not important.

glucose (Harrison 1985) in the "open" and "closed" conformations are shown in Table 1. Comparison with Fig. 2 demonstrates absolute conservation of virtually all of these residues in the hexokinases. The sole exception is Val-235, with Ser, Thr, or Cys found at the corresponding position in other hexokinases; since the interaction with Val-235 is thought to be via its carbonyl oxygen, these other residues should be able to interact in a comparable manner. It is also interesting to note that the side chains on the other residues found at this position all have relatively small side chains capable of hydrogen bonding; conceivably, interactions with the side chain occur in lieu of interactions via the carbonyl oxygen. Site-directed mutagenesis experiments have demonstrated the functional importance of these highly conserved residues. Thus, as discussed above, Arora et al. (1991) converted Ser-603, Asp-657, Glu-708, and Glu-742 in the murine type I hexokinase (corresponding to Ser-158, Asp-211, Glu-269, and Glu-302, respectively, in the yeast enzyme) to Ala, with marked detrimental effect on catalytic function. These observations make it evident that the nature of the glucose binding site, and presumably the associated conformational change, is highly conserved within the hexokinase family.

Interactions with the hydroxyl group at carbon 1 are apparently not major determinants of either binding or subsequent catalysis. Thus,

several studies of both the yeast (Salas et al. 1965; Bailey et al. 1968; Wurster and Hess 1973) and mammalian hexokinases, types I-IV (Okuda et al. 1978; Miwa et al. 1983; Meglasson and Matschinsky 1983), are generally in agreement in indicating only marginally greater affinity for the α-anomer of D-glucose. There does seem to be a slight difference between the yeast and mammalian enzymes with respect to V_{max} values, with slightly higher values reported for phosphorylation of α-D-glucose by yeast hexokinase (Salas et al. 1965; Bailey et al. 1968; Wurster and Hess 1973) while the mammalian type I, II, and III isozymes exhibited slightly higher V_{max} values with the β-anomer (Okuda et al. 1978); for mammalian type IV hexokinase, the V_{max} with the α-anomer was comparable to (Miwa et al. 1983) or slightly lower than (Meglasson and Matschinsky 1983) that seen with the β-anomer.

4.2.3 Glucose 6-Phosphate

It is evident from the comments above that only certain members of the hexokinase family exhibit sensitivity to inhibition by physiologically relevant concentrations of the product, glucose 6-phosphate, and the discussion in this section pertains only to those members, e.g., the mammalian type I, II, and III isozymes. The above discussion (and sequence comparisons shown in Fig. 2) also makes it clear that the binding sites for the substrates, glucose and ATP, are highly conserved within the hexokinases. Hence, it seems unlikely that the observed differences in susceptibility to product inhibition arise from modifications at the catalytic site, with resulting effect on binding of product at the catalytic site itself. In other words, it is not likely that glucose 6-phosphate achieves its inhibitory effect by simply sterically obstructing the catalytic site, e.g., by competing through its glucose moiety for the site required by substrate glucose. Indeed, we know of no investigators in the field who hold this view.

The above logic leads to the view that inhibition by glucose 6-phosphate results from binding of this product at a site distinct from the catalytic site per se. Based on quite different reasoning (mentioned earlier), Crane and Sols (1954) first proposed the concept of a discrete "allosteric" site for glucose 6-phosphate. Subsequent demonstration of synergistic binding of glucose 6-phosphate and hexoses (Ellison et al. 1975; Wilson 1979) clearly demonstrated that these ligands bind to discrete but interacting sites. However, the relative disposition of the regulatory and catalytic sites within the overall structure, as well as the mechanism by which inhibition results, remain in dispute.

In contrast to the situation with glucose and ATP, the nature of the amino acid residues involved in binding of glucose 6-phosphate is completely unknown. The extreme specificity of the site (Wilson and Chung 1989) has blocked efforts to label the site with affinity reagents, no crystal structure for a hexokinase-glucose 6-phosphate complex has yet been described, and there is no known "hexose 6-phosphate binding" motif that might be detected within the amino acid sequence.

Even the location of the site within the overall structure of the 100-kDa enzyme remains a matter of debate. The work of White and Wilson (1987) clearly demonstrated the presence of a glucose 6-phosphate binding site in the N-terminal half of the type I isozyme; the specificity and affinity characteristics of this site were correlated with inhibition of activity, making it reasonable to conclude that this was indeed the allosteric site. The subsequent finding that *both* the N- and C-terminal halves possessed binding sites for glucose 6-phosphate (White and Wilson 1989), even though there was only one site per intact 100-kDa enzyme (Ellison et al. 1974, 1975; Chou and Wilson 1974a) certainly complicated matters, ultimately leading to the proposal represented in Fig. 4. However, it is clear that the matter is not yet settled, e.g., Arora et al. (1993).

Jarori et al. (1990) employed nuclear magnetic resonance and a paramagnetic relaxation probe method in an effort to ascertain the distance between the glucose and glucose 6-phosphate binding sites on bovine type I hexokinase. Since the nuclear magnetic resonance signals for the anomeric protons of glucose and of glucose 6-phosphate occurred at the same position, the measurements were made on the enzyme-glucose and enzyme-glucose 6-phosphate complexes separately. Based on the effect of a paramagnetic Mn^{2+} ion, bound at a "high-affinity" site on the enzyme, on the relaxation rates for protons at the C1 position of glucose and glucose 6-phosphate, Jarori et al. estimated that these protons were between 0.3 and 1.9 nm apart. Arora et al. (1993) have used this estimate to support their contention that both the glucose and the inhibitory glucose 6-phosphate sites reside in the C-terminal half of the enzyme. However, it is not clear that the glucose 6-phosphate studied by Jarori et al. (1990) was indeed bound at the regulatory site. Although the K_i for glucose 6-phosphate is in the low μM range, Jarori et al. used glucose 6-phosphate in the 3–10 mM range. It is surely conceivable that, at such excessive levels, glucose 6-phosphate bound with low affinity at the glucose site in the C-terminal half; this was possible since glucose and glucose 6-phosphate were not present simultaneously. Indeed, Jarori and coworkers (Kenkare et al. 1985) had themselves previously reported the existence of two sites, at which

glucose 6-phosphate bound to type I hexokinase with differing affinity. If the glucose and glucose 6-phosphate binding sites interacting with the Mn^{2+} were, in fact, the *same* site, this would certainly explain why both "sites" were found in close proximity. Even if the interpretation by Jarori et al. is correct and the glucose and glucose 6-phosphate were indeed binding at the substrate and regulatory site, respectively, a separation of as much as 1.9 nm would not preclude location of the sites in distinct halves of the molecule, although admittedly this would be quite a stretch (Wilson and Smith 1985). Definition of the exact location of the glucose 6-phosphate binding site by unambiguous methods is obviously critical to resolving this matter.

Given the extensive conservation of amino acid residues implicated in binding of glucose and ATP to the N- and C-terminal halves, as well as the extensive overall similarity in sequence of the N- and C-terminal halves, it is almost a certainty that the N- and C-terminal glucose 6-phosphate binding sites, when identified, will also be found to show similarity. It should be noted, however, that there is at least one distinction between the two sites (in addition to the fact that one must be latent in the intact enzyme – see above). White and Wilson (1989) demonstrated that the isolated N-terminal half of rat type I hexokinase was protected against proteolysis as a result of binding of glucose 6-phosphate; in contrast, similar protection from binding of glucose 6-phosphate to the C-terminal half was seen only in the presence of added hexose. Since the protective effect is reasonably interpreted as a reflection of conformational changes induced by ligand binding, it is apparent that these are not equivalent in the two halves.

The requirement for simultaneous presence of glucose to evoke a glucose 6-phosphate-dependent conformational change in the C-terminal half (White and Wilson 1989) could also be interpreted as supporting the suggestion of Mehta et al. (1988), discussed above, that the allosteric site for glucose 6-phosphate is induced by prior binding of glucose, with that allosteric site being located in the C-terminus. However, leaving aside other concerns about the study of Mehta et al., noted above, this interpretation would again run into the problem that, in the presence of glucose, one would predict *two* binding sites for glucose 6-phosphate, a preexisting site in the N-terminus (White and Wilson 1987) and a glucose-induced site in the C-terminus. This is not in accord with the results of Ellison et al. (1975) *or* Mehta et al. (1988).

4.2.4 Inorganic Phosphate

At relatively low (few millimolar) concentrations, P_i antagonizes the inhibition of type I hexokinase by glucose 6-phosphate; intracellular concentrations of P_i are in this range, and hence variation in the ratio of these antagonistic ligands is generally considered to be an important factor in regulation of hexokinase activity in vivo (Ellison et al. 1975; Wilson 1978a). At higher concentrations, P_i is itself an inhibitor, competitive with ATP (Ellison et al. 1975). Ellison et al. (1975) found a single high-affinity site for binding of P_i, with a K_d of approximately 22 μM; this seems surprisingly low, considering the concentrations required to reverse inhibition by glucose 6-phosphate. The existence of a second site with much lower affinity is evident from the inhibitory effect seen at higher P_i concentrations. Binding of P_i at the high-affinity site was mutually exclusive with binding of glucose 6-phosphate at its high-affinity site (Ellison et al. 1974, 1975), providing a physical basis for the kinetic antagonism between these two ligands. White and Wilson (1990) suggested that these ligands compete for a common anion binding site in the N-terminal half of the molecule, with the low affinity site for P_i (and glucose 6-phosphate) being located in the C-terminal half of the molecule (Fig. 4).

Vowles and Easterby (1979) observed that type I hexokinase purified from pig heart differed from the type I isozyme from brain in that inhibition by glucose 6-phosphate was not reversed by P_i. This was subsequently confirmed by Shone and Fromm (1980). However, Easterby and Qadri (1981) then reported that the type I isozyme from both heart and brain *were* sensitive to reversal of glucose 6-phosphate inhibition by P_i, and that the loss of the antagonistic action of P_i occurred during storage of the enzyme. Similar results had previously been seen with the hexokinase from dog heart (Mayer et al. 1966). Thus, the ability of P_i to reverse inhibition by glucose 6-phosphate seems to be an intrinsic, if labile, characteristic of the type I isozyme. Whether the loss of this property is due to an actual inability to bind P_i or to an altered conformational response to binding of P_i has not been determined, and the chemical/structural basis for this altered response to P_i also remains unknown. Modification of sulfhydryls may be a reasonable possibility (see discussion below). Why this apparently occurs more readily with type I hexokinase from heart is an interesting question.

Type II hexokinase exhibits a somewhat different response to P_i in that this ligand does *not* antagonize the inhibition by glucose 6-phosphate, but rather, serves solely as an inhibitor, competitive versus ATP with a K_i of 2.7 mM (Lueck and Fromm 1974). The potential

physiological significance of this difference between the type I and type II isozymes in their response to P_i has been discussed previously (Lueck and Fromm 1974; Wilson 1985). There do not appear to have been any direct binding studies with the type II isozyme, and the stoichiometry of binding is therefore uncertain. Taken at face value, the kinetic results would suggest a single site with moderate affinity. There is no real basis for suggesting whether this site is located in the N- and C-terminal half of the enzyme, but by analogy with type I hexokinase, a C-terminal location might be predicted.

The effect of P_i on the type III isozyme from bovine liver (Siano et al. 1975) was similar to that on the type II isozyme, i.e., competitive inhibition versus ATP (K_i not given but apparently in the low millimolar range) and no reversal of the inhibition by glucose 6-phosphate.

Stocchi et al. (1983) reported that, unlike other mammalian erythrocytes, pig erythrocytes contain predominantly the type III isozyme, with much lesser amounts of the type II isozyme. Both isozymes were purified from this source and kinetic properties of the type III isozyme studied in detail (Magnani et al. 1983a). Inhibition by glucose 6-phosphate was not reversed by P_i; P_i itself was an inhibitor, competitive versus ATP, with a K_i of 7.8 mM. Thus, the pig erythrocyte hexokinase resembled the type II isozyme (Lueck and Fromm 1974) and the type III isozyme from bovine liver (Siano et al. 1975) in its response to P_i. However, it should be noted that, compared to the type II and III isozymes from other sources (Grossbard and Schimke 1966; Lueck and Fromm 1974; Siano et al. 1975), other kinetic properties of the pig erythrocyte enzymes (Stocchi et al. 1983; Magnani et al. 1993) were anomalous, for example, negative cooperativity in binding glucose by the type II isozyme and positive cooperativity in binding glucose by the type III isozyme.

4.3 Sulfhydryl Groups and Disulfide Bridges

Yeast hexokinase contains four Cys residues that are conserved in both isozymes A and B; these are at positions 244, 268, 398, and 404 (Fig. 2). *None* of these is conserved in yeast glucokinase or in any of the other hexokinases. Since these enzymes share a common mechanism, it is apparent that none of these Cys residues is essential for catalytic activity, in agreement with the conclusion of Colowick (1973).

The N-terminal halves of the 100-kDa mammalian hexokinases all contain seven Cys residues, at positions corresponding to residues 133,

Table 2. Conservation of Cys residues among members of the hexokinase family

	Mammalian hexokinases							
	Type II		Type III			Yeast		
Rat type I hexokinase	N	C	N	C	Type IV	Hexokinase	Glucokinase	*S. mansoni* hexokinase
Cys-133 (581)	+	+	+	+	+	–	–	–
Cys-158 (606)	+	+	+	+	–	–	–	+
Cys-217 (665)	+	+	+	+	+	–	–	+
Cys-224 (672)	+	+	+	+	+	–	–	+
Cys-237 (685)	+	+	+	+	+	–	+	–
Cys-256 (704)	+	+	+	+	+	–	–	–
Cys-368 (≈813?)	+	+	–	–	+	–	–	–
Cys-375 (823)	+	+	+	+	+	–	–	+

158, 217, 224, 237, 256, and 375 in the rat type I isozyme. These are conserved at analogous positions in the C-terminal half of the molecule (positions 581, 606, 665, 672, 685, 704, and 823, respectively, in the rat type I isozyme). The type I and II isozymes also contain another Cys in their N-terminal half (Cys-368 in rat type I hexokinase) which does not have a direct analog in the C-terminal half, although there is a Cys residue found at (rat type I) position 813 which might be considered equivalent. Type III hexokinase contains no analogous Cys in either its N- or C-terminal half. The extent to which these Cys residues are found at corresponding positions in other hexokinases is shown in Table 2. Based on the conservation within this population of Cys residues, the comparison shown in Table 2 is consistent with the view that the type IV isozyme is closely related to the 100-kDa mammalian hexokinases, and that both the 50- and 100-kDa mammalian isozymes are more closely related to the 50-kDa glucose 6-phosphate sensitive hexokinase of *S. mansoni* than they are to either the hexokinase or glucokinase of yeast. This is, of course, consistent with the evolutionary relationships discussed above (Fig. 3).

It is interesting to note that the conservation of Cys-158 (606) in rat type I hexokinase, and the equivalent residues in other hexokinases, correlates with the sensitivity to inhibition by glucose 6-phosphate; however, this appears to be merely coincidental and not functionally significant since mutation of Cys-158 or Cys-606 to Ala does not affect inhibition of the type I isozyme by the glucose 6-phosphate analog, 1,5-anhydroglucitol 6-phosphate (M. Baijal and J.E. Wilson, unpublished work).

In addition to the Cys residues discussed above, the *C-terminal* half of rat type I hexokinase contains five unique Cys residues, found

at positions 613, 628, 717, 834, and 886. The extent to which these are conserved among the other mammalian isozymes, or even between the type I hexokinases from different species, varies markedly (Fig. 2). A detailed comparison of this conservation pattern does not seem useful; suffice it to say that *none* of these Cys residues is totally conserved in the mammalian hexokinases and the *S. mansoni* enzyme; it is therefore not unexpected that corresponding Cys residues are not found in the more distantly related yeast enzymes.

In short, there is not a single Cys residue that is conserved within the hexokinase family, making it most unlikely that any Cys is essential for catalysis. Although reagents reacting with Cys residues have been shown to inactivate hexokinase from various sources (Colowick 1973; Redkar and Kenkare 1972; Chou and Wilson 1974b), it is virtually certain that this results from alteration of the enzyme's conformation or steric obstruction of the catalytic site as a consequence of modification of the sulfhydryl group(s) rather than indicating true essentiality of sulfhydryls for the catalytic mechanism (Colowick 1973; Connolly and Trayer 1979; Schirch and Wilson 1987a,b).

The reactivity of sulfhydryl groups in mammalian type I hexokinase is markedly affected by conformational changes induced by the binding of various ligands (Redkar and Kenkare 1972, 1975; Chou and Wilson 1974b; Hutny and Wilson 1990). The earlier studies were necessarily confined to determining the number of sulfhydryls reacting under particular conditions, and examining the overall kinetics of the reaction. However, cloning of the cDNA for the type I isozyme (Nishi et al. 1988; Schwab and Wilson 1988, 1989) provided a deduced sequence, making it possible to determine the relative reactivity of specific identified Cys residues within the enzyme (Hutny and Wilson 1990). The results of this study were consistent with the location of binding sites for glucose 6-phosphate and P_i discussed above. Thus, with both ligands, low concentrations markedly decreased reactivity of sulfhydryl groups in the N-terminal half of the molecule, consistent with the view that high-affinity binding sites were located in this region. Some effect on reactivity of sulfhydryls in the C-terminal half of the enzyme was also seen, attributed to the occurrence of substantial interactions, with functional importance, between the N- and C-terminal halves (see above). However, maximal effect on reactivity of sulfhydryl groups in the C-terminal half was observed only at relatively high levels of these ligands, consistent with the location of low affinity sites for glucose 6-phosphate and P_i in the C-terminal domain (White and Wilson 1990).

Disulfide bridges are relatively uncommon in intracellular proteins (Branden and Tooze 1991). It is not surprising, therefore, that yeast hexokinase is devoid of disulfides (Colowick 1973). However, this does not appear to hold for the mammalian hexokinases. The number of free sulfhydryls present in the type I isozyme from bovine and rat brain were estimated to be 12 (Redkar and Kenkare 1972, 1975) and 14 (Chou and Wilson 1974b), respectively. However, the total number of Cys residues, determined chemically, was considerably greater, and Subbarao and Kenkare (1977) estimated that three to seven disulfide bridges might be formed in the bovine type I enzyme. The disulfide content was found to be somewhat variable from one preparation to the next, suggesting that formation of at least some of the disulfide bridges occurred during purification of the enzyme. The chemical estimates of Subbarao and Kenkare (1977) were confirmed when the deduced amino acid sequences (Schwab and Wilson 1989; Griffin et al. 1991) revealed the presence of 21 Cys residues. The number and position of the (presumed) disulfide bridges remains unknown, although likely candidates could be predicted based on their proximity in the proposed structure (Schwab and Wilson 1989). For example, Cys residues at positions 217 and 224 in the N-terminus of rat type I hexokinase, and their counterparts in the C-terminal half, Cys-665 and Cys-672, are separated by a quite hydrophilic sequence which, in several cases, includes a Pro. These sequence characteristics would be consistent with a surface loop, and indeed the corresponding region in yeast hexokinase is a surface turn linking a helical region with a β-strand. It is not difficult to imagine such a structure bringing the Cys residues into proximity with resulting disulfide bond formation. Whether such linkages are formed in the reductive environment thought to exist intracellularly (Branden and Tooze 1991) is another matter.

The work of Rose and his colleagues (Murakami and Rose 1974; Rose and Warms 1982) suggests that reversible formation of (presumably) intramolecular disulfides may also occur with the type II isozyme. However, in apparent contrast to the situation with the type I isozyme (discussed above), disulfide formation is accompanied by loss of catalytic activity. Arsenite, a reagent known to complex with vicinal sulfhydryl groups, also inactivates the type II isozyme (Murakami and Rose 1974).

Apparent intramolecular disulfide bond formation, with accompanying effect on kinetic properties, has also been observed with type IV hexokinase (Tippett and Neet 1983). The distribution of the enzyme between two "kinetic states" was sensitive to concentrations of glutathione in the physiological range, leading Tippett and Neet to suggest

that regulation of the cytosolic redox potential might be a significant factor in regulation of glucokinase activity in vivo.

Thus at least three of the four mammalian isozymes appear to have the potential for formation of intramolecular disulfide bonds. However, the extent to which that potential is expressed in vivo, and the possible significance of changes in sulfhydryl/disulfide status, remain uncertain.

5 Protein Kinase Activity and Phosphorylation of Hexokinases

Several investigators have presented results indicating that yeast and mammalian hexokinases exhibit protein kinase activity and may be capable of autophosphorylation. The possible in vivo significance of these findings remains to be determined, however.

Confirming an earlier study by Menezes and Pudles (1977), Fernández et al. (1988) reported that yeast hexokinase isozyme B (isozyme PII in the nomenclature of Colowick 1973, used by these investigators) was capable of autophosphorylation. Under the in vitro conditions used, the reaction was markedly stimulated by D-xylose and required Mn^{2+}, with Mg^{2+} being much less effective. The concentrations of xylose and divalent cations (Mn^{2+} or Mg^{2+}) used by Fernández et al., presumably because they were found to be necessary for maximal effect, were 100 m*M* and 12 m*M*, respectively; these are certainly well above what might be expected in vitro. Neither the stoichiometry of phosphorylation nor the nature of the phosphorylated residue was determined. In a subsequent study, Herrero et al. (1989) reported that yeast hexokinase B had protein kinase activity, phosphorylating histone and casein. Again there was no estimate of the stoichiometry or identification of phosphorylated residues. Herrero et al. (1989) also reported that the protein kinase activity of yeast hexokinase PII was stimulated by cAMP, a surprising finding since the cAMP stimulation of well-recognized protein kinases results from binding of the cAMP to a highly conserved binding domain (Taylor et al. 1990) which is not evident within the structure of yeast hexokinase (Harrison 1985). It is worth noting that unphysiologically high concentrations of lyxose or xylose induce ATPase activity by yeast hexokinase (DelaFuente et al. 1970), but this proceeds at a rate more than a 1000-fold less than glucose phosphorylation, and it is doubtful that anyone proposes a significant in vivo role for the ATPase activity. Thus, until the effectiveness of yeast hexokinase in autophosphorylation, or as a protein kinase acting with physiologically

relevant substrates, has been determined, it seems premature to attach in vivo significance to these results (Fernández et al. 1988; Herrero et al. 1989). That yeast hexokinase may be particularly susceptible to spurious phosphorylation is suggested by the observation that yeast hexokinase, but not the mammalian type I and II isozymes, is phosphorylated by the mammalian tyrosine kinases, $pp60^{src}$ and $pp60^{csrc}$ (Coussens et al. 1985). In a preliminary communication, Seale et al. (1992) have confirmed phosphorylation of yeast hexokinase by $pp60^{csrc}$ while the mammalian type I hexokinase was very poorly phosphorylated. Seale et al. (1992) also confirmed the autophosphorylation activity of yeast hexokinase, with tentative identification of Tyr as the phosphorylated residue but stoichiometry not established; this evoked marginal effects – 28% decrease in K_m for glucose, 28% increase in V_{max} – on catalytic activity. While there is evidence that both yeast isozymes A and B may be phosphorylated in vivo, both the possible physiological consequences as well as the mechanism by which this occurs remain unclear (Vojtek and Fraenkel 1990).

Ekman and Nilsson (1988) reported phosphorylation of Ser in rat liver glucokinase by the catalytic subunit of protein kinase A. Using rather heroic concentrations of the protein kinase, stoichiometry of one phosphate per glucokinase molecule was achieved after 2 h. The data presented by Ekman and Nilsson (their Fig. 3) show no tendency to "level off" at a 1:1 stoichiometry, suggesting that further phosphorylation might have occurred with longer reaction times. Rather modest effects on glucokinase activity were seen after incorporation of one phosphate per glucokinase molecule, with about 40% decrease in V_{max} and an increase in the $K_{0.5}$ for glucose from 4 to 5.5 m*M*.

Type I hexokinase from rat brain (Adams et al. 1991) and tumor (Arora and Pedersen 1993) has also been reported to exhibit autophosphorylation, and the rat brain enzyme serves as a protein kinase with histone H2A as substrate (Adams et al. 1991). Seale et al. (1992) also found that the tumor and bovine brain type I isozymes served as substrates for cAMP-dependent protein kinase. In none of these studies has the stoichiometry of phosphorylation been determined. According to the recent report of Arora and Pedersen (1993), *both* the N- and C-terminal halves of murine type I hexokinase are capable of autophosphorylation at Tyr residues. This is particularly surprising since, as discussed above, the N-terminal half lacks glucose phosphorylation activity (confirmed by Arora et al. 1993, and Arora and Pedersen 1993); evidently the requirements for use of ATP as a substrate for autophosphorylation are less restrictive than those for glucose phosphorylation.

Arora and Pedersen (1993) also found that glucose had no effect on the autophosphorylation activities of the yeast and murine type I hexokinases; this is in contrast to the results of Adams et al. (1991) who reported that autophosphorylation was blocked in the presence of glucose.

While autophosphorylation as well as the protein kinase activity attributed to hexokinases are interesting, it is evident that further characterization is required before the possible in vivo significance of these phenomena can be assessed. Thus far, there has been no evidence that phosphorylation has any substantial effect on activity or other properties of the enzyme, and phosphorylation of the mammalian enzymes in vivo has not been demonstrated.

A very interesting exception to the last statement has been found in sperm. A 95-kDa phosphotyrosine-containing protein (p95), located in the sperm membrane and serving as a receptor for interactions with the egg zona pellucida, has, based on sequence similarity, been identified as hexokinase (Kalab et al. 1994). Mori et al. (1993) have recently described three mRNAs that appear to code for unique forms of hexokinase, possibly including p95, in spermatogenic cells. These mRNAs would encode hexokinase forms having sequences in the N-terminal region that are distinct from the somatic form (Arora et al. 1990a). The remainder of the coding sequence (about 2700 nucleotides) was almost identical to that reported previously for the mouse type I isozyme (Arora et al. 1990a), with only 15 (unspecified by Mori et al.) nucleotide differences, leading to changes in deduced amino acid sequence at two positions. If there is indeed a single gene for the type I isozyme, as appears to be the case (Wigley and Nakashima 1992; Daniele et al. 1992), the spermatogenic cell specific mRNAs presumably result from alternative splicing and the reported nucleotide differences may be due to sequencing errors or polymorphisms. From comments above it should be evident that modifications confined to the extreme N-terminal region are expected to leave hexokinase activity intact. Although intrinsic hexokinase activity of p95 does not appear to have yet been demonstrated, it is interesting to note that Katzen (1967) detected a unique "sperm type" hexokinase activity, migrating on starch gels with a mobility slightly less than that of the type I isozyme. Could it be p95?

It is also interesting that Kalab et al. (1994) reported that the migration of p95 on denaturing gels was different under reducing and nonreducing conditions, suggesting the presence of disulfide bridges in the molecule (see comments above).

6 Kinetic Mechanism of Hexokinases

The adage "kinetics can never prove, but only disprove, a mechanism" is well known. Inherent uncertainty is characteristic of kinetics. There could hardly be a better illustration of this than that provided by the long-standing effort to define the kinetic mechanism(s) of the hexokinases. From the late 1960s and into the 1980s, a series of publications describing kinetic studies of the yeast and mammalian hexokinases emanated from various laboratories. Despite these efforts, it could hardly be said that the kinetic mechanism for any of the hexokinases has been established unequivocally. Indeed, the ability to interpret, re-interpret, and adapt the results of kinetic studies to quite different mechanisms has been rather amazing. Activity in this area now seems to have subsided, perhaps due to exhaustion, loss of interest, or simply uncertainty about what to do next.

What does seem to be universally accepted is that these enzymes operate by a sequential mechanism, i.e., formation of a ternary enzyme-glucose-ATP-Mg^{2+} complex is required. It is noteworthy that early kinetic studies with mammalian isozymes (Fromm and Zewe 1962a; Hanson and Fromm 1965; Copley and Fromm 1967) were interpreted as excluding a sequential mechanism, followed shortly thereafter by additional kinetic studies from the same laboratory (Hanson and Fromm 1967; Fromm and Ning 1968) leading to the opposite conclusion. This is not meant as criticism, but simply to indicate that, from early on, kinetic studies with hexokinases seem to have been plagued by complications that forced continued reevaluation of previous conclusions and, not infrequently, led to debate of an intensity that one might have thought would be reserved for more momentous matters.

It is also quite clear that the mammalian type IV isozyme is kinetically distinct from the other mammalian isozymes and the yeast isozymes. Given the extensive sequence similarity between all these enzymes (Fig. 2), this is perhaps surprising, but it demonstrates that rather subtle changes might underly functional changes that adapt the isozymes for quite different metabolic roles. This statement makes the perhaps arguable assumption that kinetic properties studied in vitro have relevance to in vivo function.

6.1 Yeast Hexokinase

Colowick reviewed (1973) earlier kinetic studies of yeast hexokinase, which included claims that the enzyme operated by an ordered

sequential mechanism, with glucose binding first (e.g., Noat et al. 1968), as well as work interpreted to indicate that formation of the ternary complex occurred by random addition of substrates (e.g., Fromm and Zewe 1962b; Fromm et al. 1964; Rudolph and Fromm 1970, 1971). It is not our intention to reiterate Colowick's analysis here; we will merely note that Colowick concluded that "all of the data become *reasonably compatible* [emphasis added] if one grants that the process may be random in the sense that either substrate may be the first to add and either [product] may be the last to leave, but that there are *quantitative* differences that make one pathway predominate over another." This view has apparently endured (Danenberg and Cleland 1975; Viola et al. 1982; Taylor et al. 1983), although there seems to be a rather wide range of opinions as to the extent to which the binding of glucose represents the initial event in the catalytic cycle. Thus, Dorgan and Schuster (1981) speak of a random mechanism with a "slightly preferred, though not mandatory, binding order" whereas Cleland and coworkers have described the mechanism as "somewhat random" (Viola et al. 1982) or "essentially ordered with glucose adding prior to MgATP" (Taylor et al. 1983) or "a strong preference for sugar binding first" (Jones et al. 1991). The relative importance of the ordered addition pathway, glucose adding first, has also been said to depend on substrate levels, with the majority of the flux going through the "glucose first" path when substrates are present at concentrations near K_m while, at "physiological" levels of substrate, the flux through both paths (either glucose or ATP adding first) may be comparable (Danenberg and Cleland 1975).

Surely most studies have led to the view that, at a minimum, there is a strong propensity for the reaction to go through the ordered path, with glucose adding first. This seems intuitively satisfying in that it imparts functional significance to the conformational change induced by binding of glucose (Anderson et al. 1978a,b; Bennett and Steitz 1980; Harrison 1985), which markedly enhances the binding of ATP (DelaFuente and Sols 1970; DelaFuente et al. 1970; Roustan et al. 1974; Peters and Neet 1978; Viola et al. 1982; Woolfitt et al. 1988). As noted above, Shoham and Steitz (1980) suggested that cleft closure, induced by binding of glucose, might enhance binding of ATP by bringing residues from both small and large lobes into position for interaction with the nucleotide. If the latter interactions could also occur prior to binding of glucose, it is not difficult to envisage that this might impede access to the glucose binding site deep in the cleft. Thus, a preferred order, with glucose first, seems a reasonable expectation. However, one could imagine that an enzyme-ATP complex, in which the nucleotide

was interacting only with the "primary" site in the large lobe (Shoham and Steitz 1980) and thus not grossly hindering access of glucose, could be a productive intermediate; hence, an absolute requirement for ordered addition would not be seen.

As discussed above, there is some reason to consider the glucokinase from *Z. mobilis* (Barnell et al. 1990), despite its smaller size, as homologous to eukaryotic hexokinases. Whether this is true for hexokinases from other prokaryotes remains to be determined. Nonetheless, it is interesting to note that kinetic studies of hexokinases from *Streptococcus mutans* (Porter et al. 1982) and *Bacillus stearothermophilus* (Ishikawa et al. 1987) have led to the conclusion that these enzymes function by an ordered mechanism, glucose adding first and glucose 6-phosphate leaving last.

6.2 Mammalian Hexokinases, Types I–III

The mammalian type I and II isozymes have been the subject of extensive kinetic studies, and these provide the basis for most of the discussion here. Studies with the type III isozyme have been much more limited, probably due to the fact that this isozyme is present in quite low amounts in most tissues and not easily purified in substantial amounts. Studies by Grossbard and Schimke (1966) and Radojkovic and Ureta (1987) indicate that the rat type III isozyme is generally similar to the rat type I and II isozymes in its kinetic properties, and the same is true for the type III isozyme from bovine liver (Siano et al. 1975). Perhaps the most striking difference is that type III hexokinase is considerably less sensitive to inhibition by glucose 6-phosphate, the K_i being three- to tenfold higher than the K_is for the type I or II hexokinases. In addition, type III hexokinase shows considerably higher affinity (lower K_m) for substrate glucose, and substrate inhibition at higher but still physiological glucose concentrations. Although more complex kinetic explanations might be offered (Siano et al. 1975), the latter property suggests the presence of a second, lower affinity site for binding of glucose, and the latent glucose binding site thought to exist in the N-terminal half of the type I isozyme comes to mind (see discussion above); perhaps this site is not totally latent in type III hexokinase and serves a regulatory role.

As mentioned above, pig erythrocytes are reported (Stocchi et al. 1983) to contain an enzyme classified as type III hexokinase based on its charge properties; however, the kinetic properties of this hexokinase

(Magnani et al. 1983a) are quite dissimilar from those reported for the type I, II, and III isozymes from other sources, and this rather anomalous hexokinase of pig erythrocytes is not included in this more general discussion.

Colowick (1973) reviewed the then-limited literature on kinetics of mammalian hexokinases and concluded that these are similar to the yeast enzyme, with ordered addition of glucose then ATP being the preferred kinetic pathway. Although also favored by other investigators (e.g., Bachelard et al. 1971; Easterby and O'Brien 1973), Fromm and his colleagues have been particularly persistent advocates of a random addition mechanism for the type I (Ning et al. 1969; Purich and Fromm 1971; Ellison et al. 1975; Solheim and Fromm 1983), type II (Lueck and Fromm 1974; Ganson and Fromm 1985; Bass and Fromm 1987), and type III (Siano et al. 1975) isozymes. Ganson and Fromm (1985) did, however, conclude from equilibrium isotope exchange studies with the rat type II isozyme that the path with addition of glucose first is favored; similar studies have not been done carried out with the type I or type III isozymes.

Unlike the situation with yeast hexokinase (DelaFuente and Sols 1970; DelaFuente et al. 1970; Roustan et al. 1974; Viola et al. 1982; Woolfitt et al. 1988), binding of ATP (and analogs) *can* occur in the absence of glucose with affinities comparable to those estimated by kinetic methods (Wilson 1978a; Baijal and Wilson 1982). Whether enzyme-ATP is a productive complex, i.e., can add glucose and go on to product, is another question. In principle, one might use initial velocity or isotope exchange methods to distinguish a truly random mechanism from an ordered mechanism with formation of a nonproductive binary complex. If glucose added first in the pathway leading to product, but the second substrate, ATP, could also bind free enzyme to yield a nonproductive complex, substrate inhibition by ATP is predicted at low concentrations of glucose (Purich et al. 1977). However, Purich et al. (1977) showed that this may depend on the k_{cat} value of the enzyme. The k_{cat} for type I hexokinase is approximately 100 s^{-1} (Wilson 1989), and that for the type II isozyme perhaps two- to fourfold higher (Holroyde and Trayer 1976; Qadri and Easterby 1980). Based on the analysis of Purich et al. (1977), one might expect substrate inhibition to just barely be detectable with the type I isozyme. Although some suggestion that this occurs may be seen in the results of Fromm and Zewe (1962a) and Copley and Fromm (1967), the absence of reported substrate inhibition by ATP in subsequent kinetic studies suggests that this was spurious. Moreover, there was *no* suggestion of substrate inhibition

of the type II isozyme (Hanson and Fromm 1967) even though the higher k_{cat} should have made this more evident (Purich et al. 1977). Thus these results, as well as equilibrium isotope exchange studies (Ganson and Fromm 1985), do not support an ordered mechanism with glucose binding first and formation of a nonproductive enzyme-ATP complex.

Inhibition patterns, obtained with either products or substrate analogs, are also expected to be of use in deciding between possible mechanisms. Here too, life with hexokinase has not been simple. Part of the problem has been that interpretation of the inhibition patterns themselves has not always been unambiguous. For example, inhibition of rat type II hexokinase by AMP was originally described as noncompetitive (Hanson and Fromm 1967) but subsequently said to be competitive (Ganson and Fromm 1985). Although inhibition of both the rat (Grossbard and Schimke 1966; Purich and Fromm 1971) and bovine (Copley and Fromm 1967; Ning et al. 1969) type I hexokinase by ADP was described as noncompetitive versus glucose, the lines in the diagnostic double reciprocal plots (1/v versus 1/glucose) departed only slightly from the parallel pattern characteristic of uncompetitive inhibition, and in fact in earlier studies with the bovine brain enzyme, inhibition had been said to be uncompetitive (Fromm and Zewe 1962a); this is significant because an uncompetitive pattern would be consistent with an ordered addition mechanism. Even established product inhibition patterns have not been interpreted without ambiguity, supporting either ping pong (Fromm and Zewe 1962a; Copley and Fromm 1967) or sequential (Fromm and Ning 1968; Ning et al. 1969) mechanisms, depending on other circumstances. See Gregoriou et al. (1983) for additional comments in a similar vein.

The inhibition seen with ADP, noncompetitive versus ATP (Fromm and Zewe 1962a; Grossbard and Schimke 1966; Copley and Fromm 1967; Ning et al. 1969; Kosow and Rose 1970; Purich and Fromm 1971) has been especially problematic since it does not fit neatly into any of the mechanisms favored by various proponents, forcing creativity. Ning et al. (1969) postulated the existence of an "allosteric" site, to which ADP and other nucleotides could bind in addition to binding at the substrate ATP site; further support for this was found in the observation that inhibition exhibited a higher order (implied but not really shown to be second order) dependence on ADP concentration, taken to indicate the existence of more than one site for this ligand. While it may be true that this could explain the results, these authors were forced to postulate that this allosteric site could bind "ADP, AMP, GTP, GDP, ITP, IDP, IMP, 3′,5′-cyclic AMP, and 3-phosphoglycerate" but could

not bind ATP. Surely it is difficult to believe that such an astonishing lack of specificity could, at the same time, be accompanied by selective exclusion of ATP. Kosow and Rose (1970) proposed an alternative explanation for the noncompetitive (versus ATP) inhibition by ADP. They attributed this to slow dissociation of glucose 6-phosphate from the enzyme, allowing ADP to interact with an enzyme-product complex; it is readily shown that this could lead to noncompetitive inhibition without the necessity to postulate binding at any site other than the substrate (or product, depending on your point of view) site. Kosow and Rose also presented isotope exchange results that were "inconsistent with the rapid equilibrium mechanism, as well as with an allosteric site of ADP action", as proposed by Ning et al. (1969). Purich and Fromm (1971) then responded that Kosow and Rose's results "cannot be rationalized in terms of a single binding site" for ADP. Such flagrantly contradictory assertions are indeed challenging. It is not apparent to this writer that this matter has ever been resolved in any satisfactory manner, and it seems useful to give further consideration to reevaluating these kinetic studies, and perhaps conducting additional kinetic studies, in light of more recent work on structure of the enzyme. For example, the notion of an "allosteric" site binding ADP and other nucleotides (but including ATP) seems much less speculative now that the existence of nucleotide binding sites in *both* N- and C-terminal halves of the type I isozyme has been established (White and Wilson 1989, 1990). Also, Purich and Fromm (1971) "ruled out" an ordered mechanism, glucose binding first, based on the expectation that "the product of the first substrate in an ordered mechanism should be a competitive inhibitor with respect to that substrate", whereas glucose 6-phosphate is a noncompetitive inhibitor versus glucose (Grossbard and Schimke 1966; Copley and Fromm 1967); this expectation was based on the assumption that both glucose and glucose 6-phosphate compete for a common binding site, which is not the case (see above).

Kinetic methods have also been directed at determining the mechanism by which glucose 6-phosphate inhibits the enzyme. As discussed above, there is considerable evidence to support the view, first proposed by Crane and Sols (1954), that inhibition by glucose 6-phosphate results from binding of this ligand at a discrete regulatory site on the enzyme. Thus, the competitive (versus ATP) inhibition (Grossbard and Schimke 1966; Copley and Fromm 1967) could be considered to result from binding of the inhibitor at a site in the N-terminal half (White and Wilson 1987), inducing conformational changes that preclude binding of ATP at the catalytic site in the C-terminal half (Baijal and Wilson 1982; White

and Wilson 1990). Fromm and colleagues, however, have suggested that the competitive inhibition results from competition of the γ-phosphate of ATP and the 6-phosphate of glucose 6-phosphate for a common anion binding site within the catalytic region (Casazza and Fromm 1976). In this view, after phosphorylation the glucose moiety is displaced to an adjacent site (but with the phosphate staying in place), permitting the original hexose binding site to accept another ligand, i.e., permitting formation of an enzyme-glucose-glucose 6-phosphate ternary complex (Ellison et al. 1975; Wilson 1978a). Solheim and Fromm (1981) examined the reverse reaction and determined the K_m for glucose 6-phosphate to be about 25 μM, i.e., similar to the K_i for glucose 6-phosphate as an inhibitor of the forward reaction – and, one might add, to the K_d for binding of glucose 6-phosphate to the intact enzyme (Ellison et al. 1974, 1975; Chou and Wilson 1974a; White and Wilson 1987). Solheim and Fromm concluded that their results were "consistent only with the high-affinity site for glucose-6-P on brain hexokinase being the active site." Admittedly this is a strong argument, but strong arguments for other points of view and based on kinetic data have proven to be open to alternative interpretation. Location of a functional high-affinity site for glucose 6-phosphate in the catalytic C-terminal domain (White and Wilson 1989) is difficult to reconcile with the existence of a high-affinity binding site for glucose 6-phosphate in the N-terminal half (White and Wilson 1987) and with binding studies showing a single site for glucose 6-phosphate (Ellison et al. 1974, 1975; Chou and Wilson 1974a). Regardless of the topological interpretation, the results of Solheim and Fromm (1981) provide further argument against the suggestion by Mehta et al. (1988) that the high-affinity site for glucose 6-phosphate is induced by prior binding of glucose; there was no significant glucose present under the conditions used by Solheim and Fromm to study the reverse reaction.

As one final example of the tortured history of kinetic studies with the 100-kDa mammalian hexokinases, we note the work of Gregoriou et al. (1983). Acting upon the suggestion of Colowick (1973), these authors applied the flux ratio isotope exchange method to the type II isozyme from rat muscle. The flux ratio method was touted as "less ambiguous" than product inhibition patterns as a guide to deducing kinetic mechanisms. The results were said to "provide for the first time unambiguous evidence that glucose and ATP bind to hexokinase II in compulsory order." Ganson and Fromm (1985) disagreed, producing equilibrium isotope exchange results and inhibition studies in support of a steady-state random addition mechanism, and criticizing, largely

on technical grounds, the experiments of Gregoriou et al. (1983). The latter authors responded (Gregoriou et al. 1986), offering a point-by-point response to the criticism of Ganson and Fromm, challenging and pointing out inconsistencies in some of the points raised by the latter authors, and concluding that "with plausible values assumed for the kinetic constants," their proposed (ordered) mechanism led to predicted behavior "that closely resembles the experimental inhibition experiments that have been claimed against it." Not to be outdone, Bass and Fromm (1987) provided further technical criticism of the flux ratio experiments of Gregoriou et al. (1983), concluding that their proposed mechanism was "at variance with a large amount of kinetic data ... in the literature" and conflicted with equilibrium isotope exchange results reported by Gregoriou et al. (1983) themselves. There the matter rests.

Studies of the 100-kDa mammalian hexokinases have provided a marvelous illustration of the uncertainty inherent in kinetics. Some eminent kineticists have been involved in these studies. Given *their* inability to come to consensus on a kinetic mechanism, it seems likely that mechanistic complexities associated with the hexokinases are considerably greater than those encountered with "normal" enzymes – though a deceptively simple but long-overlooked explanation for the seeming contradictions should not be excluded. Some may view this as a challenge; others may view it as a frustration that more than 30 years of kinetic studies have left us in such a state.

6.3 The Type IV Isozyme of Mammalian Hexokinase

Unlike the other mammalian isozymes, type IV hexokinase does not obey Michaelis-Menten kinetics with glucose as varied substrate, although normal Michaelis-Menten behavior is seen with ATP as varied substrate (Storer and Cornish-Bowden 1977). Since glucokinase functions as a monomer (Cárdenas et al. 1978), the sigmoidal dependence of rate on glucose concentration cannot be explained in terms of interactions between multiple binding sites, as with classical oligomeric enzymes (Monod et al. 1963). Based on concepts developed by Ricard et al. (1974), Storer and Cornish-Bowden (1977) proposed a "mnemonic" mechanism to explain this unusual kinetic behavior. Briefly, it was suggested that glucokinase operated by an ordered mechanism, glucose binding first. The enzyme was suggested to exist in two conformations, E and E′, with E being the catalytically competent conformation and having a higher affinity for glucose. At low ATP concentrations, bind-

ing of the second substrate became rate limiting, allowing time for E and E′ to equilibrate; kinetically, this would result in the system functioning according to Michaelis-Menten kinetics since the equilibrated enzyme forms would mimic a single enzyme. However, at higher concentrations of ATP, binding of ATP would not be rate limiting and equilibration between E and E′ would not occur; apparent cooperativity with respect to glucose would then arise as a result of changes in the relative amounts of E and E′ as glucose concentrations increased, with higher glucose "trapping" the enzyme in the catalytically competent E form.

Based on product inhibition patterns, Storer and Cornish-Bowden (1977) proposed ordered release of products, with ADP being the last to leave. Gregoriou et al. (1981) presented results of flux ratio isotope exchange experiments that were considered to support the mnemonical mechanism of Storer and Cornish-Bowden (1977) except that the order of release of products was reversed, with glucose 6-phosphate now suggested to leave last. This obviously necessitated some reinterpretation of product inhibition patterns, not unprecedented in kinetic studies of the hexokinases.

Although the flux ratio results indicated that an ordered path of substrate addition, glucose first, is strongly favored during steady state, equilibrium isotope exchange experiments indicated the possibility of significant flux through an alternative path, with ATP binding before glucose. As noted above, similar results have been seen with the type II isozyme (Gregoriou et al. 1983; Ganson and Fromm 1985). This illustrates a significant difference, mentioned by Gregoriou et al. 1981, and earlier by Colowick (1973), between the flux ratio and equilibrium isotope exchange methods; the former may indicate preferred (ordered) paths that might not be evident from equilibrium exchange. In other words, even though alternative paths may be available, and functional under equilibrium conditions, only one may contribute significantly under the steady-state conditions used for flux ratio measurements. It would also seem that the latter are most appropriate for integration with other types of kinetic measurements, for example, product inhibition studies performed under steady-state conditions.

Storer and Cornish-Bowden (1977) had considered alternative mechanisms that might explain the cooperativity seen with glucokinase, but decided that their mnemonic mechanism was to be preferred. Other alternatives suggested by Pettersson (1986a,b) were discussed by Cornish-Bowden and Storer (1986) but found to be in conflict with various experimental observations. Cárdenas et al. (1984b) proposed a "slow

transition" model that is similar to the mnemonic mechanism in that it is an ordered mechanism, glucose binding first, with cooperativity attributed to conformational changes influenced by binding of the hexose. Subsequent kinetic studies by Pollard-Knight and Cornish-Bowden (1987) were considered compatible with either the mnemonic or slow transition model. The major difference between these proposals, as noted by Cárdenas et al. (1984b), is that the mnemonic model postulates a single conformational state ("E" in the discussion above) for the enzyme-glucose complex, while the slow transition model allows for two different conformational states in both the liganded and unliganded forms. Further support for the slow transition model has been provided by work of Neet and his colleagues (Neet et al. 1990; Lin and Neet 1990), and this would thus seem to be the most generally accepted mechanism at the present time. Implicit in this is acceptance of an ordered addition of substrates, glucose first.

7 Mammalian Hexokinase Function in the Physiological Context

Since phosphorylation of glucose plays a fundamental role in the metabolism of virtually all life forms, hexokinases are widely distributed throughout the biological spectrum. Although their obvious function is to produce glucose 6-phosphate as a substrate for further metabolism, catalytic capability per se cannot be their sole raison d'être. Were this to be the case, it would indeed be difficult to rationalize the existence of multiple isozymes, all capable of catalyzing this same reaction, within the same organism and even within the same cell (see below). It is reasonable to infer that the various isozymes possess properties other than basic catalytic ability that tailor them to play unique roles in cellular metabolism. Furthermore, although studies with prokaryotic organisms are rather limited, it seems to be the case that these generally contain a single form of the enzyme (e.g., Kamel et al. 1966; Hengartner and Zuber 1973; Porter et al. 1982; Scopes et al. 1985). In contrast, even unicellular eukaryotes are found to possess two or more isozymes (Colowick 1973; Reeves et al. 1967). It is not unreasonable to suspect that the unique functions of hexokinase isozymes may be related to the more complex cellular architecture of eukaryotic organisms, and indeed there is considerable evidence to support the view that the physiological role of hexokinase isozymes may be intimately related to the structural context within which glucose phosphorylation occurs.

The existence of three forms of hexokinase in wild type *S. cerevisiae* has been discussed above. Lobo and Maitra (1977) studied the growth and metabolism of mutant strains possessing only a single form of the enzyme with the hope of determining physiological roles that might be uniquely associated with the various forms but were unable to find any significant differences that could be attributed to the particular isozyme present. Although a possible role for glucokinase in transport of glucose into the cell has been suggested, this is not clearly established (Clifton et al. 1993). It should also be noted here that multiple isozymes of hexokinase may not always be the case in yeast since the work of Prior et al. (1993) suggests the existence of a single hexokinase, coded by the *RAG5* gene, in *Kluyveromyces lactis*. Like Clifton et al. (1993), Prior et al. (1993) suggested that hexokinase was somehow involved in glucose transport since the latter function was altered in *RAG5* mutants. Prior et al. (1993) also made the interesting observation that transformation of a *S. cerevisiae* mutant, in which the normal isozymes of hexokinase and glucokinase were not expressed, with the *RAG5* gene resulted in restoration of glucose phosphorylating activity but *not* the glucose repression of invertase activity seen with wild-type *S. cerevisiae*. Prior et al. (1993) suggested that the glucose repression phenomenon might depend not only on hexokinase activity per se but on physical interaction of the enzyme with other cellular components; presumably the *RAG5* gene product, though capable of glucose phosphorylation, could not provide the interactions required for glucose repression.

Thus, although there are some intriguing possibilities, the physiological significance that might be attributed to the existence of multiple forms of hexokinase in yeast remains unclear. While it may validly be argued that this is also the case with the mammalian hexokinases, it is certainly true that studies of the latter have been much more extensive and have provided at least a reasonable basis for speculation about the relationship between isozyme type, cellular location, and physiological function. For this reason the present discussion focuses on the mammalian isozymes. Of these, the type IV isozyme is, in many respects, markedly different from the 100-kDa isozymes, and we therefore discuss the type IV isozyme as a separate entity in a subsequent section.

7.1 Intracellular Distribution

The distribution of metabolites, macromolecules, and organelles in eukaryotic cells is neither random nor uniform (Clegg 1988; Lynch and

Paul 1988; Jones and Aw 1988; Aw and Jones 1988). Few would believe that this has no metabolic significance.

7.1.1 Binding of Hexokinase to Mitochondria

7.1.1.1 Occurrence and Physical Basis

Brain contains virtually exclusively the type I isozyme (Grossbard and Schimke 1966; Katzen et al. 1970; Wilson 1985). Crane and Sols (1953) observed that 80%–90% of the hexokinase activity in brain homogenates, and lesser amounts in homogenates of other tissues, was in a particulate fraction, suggested but not shown to be the mitochondria. Subsequent centrifugal fractionation studies from a number of laboratories (see Wilson 1985) led to the conclusion that hexokinase is indeed associated with mitochondria in a number of mammalian tissues. It is interesting to note that binding of hexokinase to mitochondria in plants has also been reported (e.g., Tanner et al. 1983; Miernyk and Dennis 1983; Cosio and Bustamante 1984; Dry et al. 1983; Stewart and Copeland 1993).

Craven and Basford (1969) showed that the distribution of type I hexokinase in brain, determined immunohistochemically at the light microscopic level, was consistent with a mitochondrial location. Subsequent immunohistochemical localization at the electron microscopic level (Kao-Jen and Wilson 1980) demonstrated intense staining at the periphery of mitochondria in various cerebellar cell types, with additional staining seen in cytoplasmic regions. Confirming the earlier microdissection studies of Lowry et al. (1961), Wilkin and Wilson (1977) and Simurda and Wilson (1980) provided histochemical and immunofluorescence results demonstrating that hexokinase is present at high levels in mitochondria-rich regions of retina, and also in neural regions having a high concentration of nerve endings. The latter observation is in agreement with other results (Wilson 1972a; Snyder and Wilson 1983) indicating that synaptosomal mitochondria have relatively high hexokinase activity. Lynch et al. (1991) have used confocal imaging techniques to determine the distribution of hexokinase in astrocytes cultured from rat brain. Based on quantitative analysis of immunofluorescence distribution, they estimated that approximately 70% of the hexokinase is associated with mitochondria in these cells. This is much higher than the 20% estimated by centrifugal fractionation of astrocyte homogenates (Lusk et al. 1980). Lynch et al. (1991) suggested that this might result from release of the enzyme during the fractionation procedure. The results of Lynch et al. (1991) provide a

clear illustration of the potential advantages of in situ localization methods compared to classical subcellular fractionation (also see comments below on intracellular location of type III hexokinase).

At least with brain mitochondria, hexokinase may be preferentially bound at contact sites, regions in which the inner and outer mitochondrial membranes become closely apposed – perhaps partially fused – and which are thought to have particular functional significance in interactions between intra- and extramitochondrial compartments (Nicolay et al. 1990; Brdiczka 1991). Thus, a membrane fraction with characteristics (presence of enzyme markers for both inner and outer mitochondrial membranes) expected of contact sites and enriched in hexokinase was obtained after osmotic lysis of brain mitochondria (Kottke et al. 1988; Adams et al. 1989). Hexokinase activity was also associated with "contact site" fractions from liver and kidney, but based on relative specific activity the outer membrane fraction was more enriched, suggesting that there was no preferential binding at contact sites of mitochondria from the latter tissues (Adams et al. 1989). Kottke et al. (1988) also presented results of immunogold labeling studies which they believed to support preferential location of the enzyme at contact sites; while labeling at the mitochondrial periphery – but *not* at inner membrane sites (see Magnani et al. 1993) – was evident, this reviewer found it difficult to unambiguously distinguish contact site and non-contact site regions in the micrographs; hence, the analysis may be somewhat ambiguous. Further support for the preferential binding of hexokinase at contact sites in heart (Aubert-Foucher et al. 1984) and brain (Dorbani et al. 1987; Kottke et al. 1988; BeltrandelRio and Wilson 1992a) mitochondria comes from studies showing that a major portion, 50%–80%, of the bound enzyme, is located in digitonin-resistant membrane regions thought to correspond to contact sites (Brdiczka 1991). Manipulation of conditions thought to affect formation of contact sites has been reported to affect binding of type I hexokinase to liver mitochondria (Wicker et al. 1993).

The outer mitochondrial membrane has been shown to be greatly enriched in type I hexokinase binding ability (Rose and Warms 1967; Kropp and Wilson 1970; Felgner et al. 1979). Based on a units hexokinase bound per milligram of membrane protein basis, hexokinase binding (nonspecifically?) to rat liver inner mitochondrial and microsomal membranes and to human erythrocyte membranes was approximately 100-fold less than that seen with rat liver outer mitochondrial membranes (Felgner et al. 1979). A "hexokinase binding protein" was isolated from the outer membrane of rat liver mitochondria (Felgner et al.

1979), and subsequently shown (Fiek et al. 1982; Lindén et al. 1982) to be identical to the pore-forming protein ("porin") responsible for forming the channel through which metabolites traverse the outer mitochondrial membrane; the metabolic significance of this location is discussed further below. Lindén et al. (1984) reported that in rat liver porin is located only in the outer mitochondrial membrane. More recent work suggests that the mitochondrial porin may be but one member of a family of related channel forming proteins, widely expressed in mammalian tissues; some of these may be found in other cellular membranes (Thinnes 1992; Bureau et al. 1992; Blachly-Dyson et al. 1993; Ha et al. 1993). Blachly-Dyson et al. (1993) expressed two isoforms of human porin in yeast, and found that one exhibited hexokinase binding activity while the other did not.

The outer mitochondrial membrane channel is thought to be formed by a single porin monomer. Proposed structures (De Pinto et al. 1991; Thomas et al. 1993) are in agreement in suggesting formation of the pore as a β-barrel, with amphipathic character of the β-strands providing the basis for formation of a hydrophilic channel within the hydrophobic core of the membrane. The proposed structures differ in certain aspects, including the number of β-strands thought to comprise the transmembrane barrel, 12 strands (Thomas et al. 1993) or 16 strands (De Pinto et al. 1991), and the disposition of an N-terminal helix either within (Thomas et al. 1993) or at the cytoplasmic surface of (De Pinto et al. 1991) the membrane. The pore exhibits voltage-dependent properties, with some modest selectivity for anions; hence another common name for this protein is VDAC (Voltage-Dependent Anion Channel). Specific residues involved in determining the electrophysiological properties of the channel have been suggested by site-directed mutagenesis experiments (Thomas et al. 1993).

Nakashima et al. (1986) reported that binding of hexokinase to hepatoma mitochondria was inhibited by treatment of the mitochondria with N,N′-dicyclohexylcarbodiimide (DCCD), and porin was shown to be a site for reaction with this reagent. De Pinto et al. (1993) identified the reactive residue as Glu-72 in bovine heart porin. Based on the model proposed by De Pinto et al. (1991), this would be located in the fourth (of 16) transmembrane β-strands and would be oriented toward the hydrophobic core of the membrane; this is consistent with a hydrophobic environment being necessary to evoke selective reactivity of DCCD with the glutamate side chain.

Rose and Warms (1967) first noted that treatment of type I hexokinase with chymotrypsin resulted in loss of the ability to bind to mito-

chondria, but had no effect on catalytic activity. Kurokawa et al. (1983) showed that chymotrypsin treatment resulted in loss of the ability of rat type I hexokinase to bind both to mitochondria to the hydrophobic matrix, Phenyl-Sepharose. The latter authors suggested that the bindable form of hexokinase (i.e., prior to chymotrypsin treatment) had a hydrophobic region responsible for specific interaction with mitochondria. Polakis and Wilson (1985) showed that chymotrypsin selectively removed a hydrophobic sequence at the N-terminus of the enzyme that was absolutely critical for binding. Endogenous proteases, likely of lysosomal origin (Rose and Warms 1967; Polakis and Wilson 1985; Yokoyama-Sato et al. 1987; Okazaki et al. 1992b), in brain and other tissues can modify this N-terminal segment, resulting in loss of binding ability. Purification procedures designed to minimize this artifactual modification during purification of the type I isozyme from rat brain have been described (Polakis and Wilson 1982; Wilson 1989).

Xie and Wilson (1988) showed that the hydrophobic N-terminal segment was inserted into the hydrophobic core of the membrane when hexokinase was bound to mitochondria. The results of Gelb et al. (1992) suggest that the first 15 residues of the type I hexokinase sequence may be not only necessary but sufficient to target hexokinase to the mitochondria. A chimeric protein, with this N-terminal sequence fused to a chloramphenicol acetyltransferase reporter sequence, was shown to bind in a DCCD-sensitive manner to rat liver mitochondria; binding of the chimeric protein was competitive with authentic rat type I hexokinase. The latter observation was taken (probably correctly) to indicate specific binding, but this was not directly demonstrated by showing absence of significant binding to other (e.g., microsomal) membranes. De Pinto et al. (1993) suggested that insertion of the hydrophobic N-terminal segment into the hydrophobic core might bring a positively charged Lys residue, Lys-15 (Fig. 2), into position for electrostatic interaction with the DCCD-reactive Glu-72 of porin. However, it is unlikely that this is a determinative factor in binding of hexokinase since two isoforms of human porin characterized by Blachly-Dyson et al. (1993) had identical sequences in this region yet differed markedly in their ability to induce binding of rat type I hexokinase to mitochondria.

It might be noted here that p95, the unique form of type I hexokinase (Kalab et al. 1994) found in sperm (see comments above), apparently lacks the N-terminal sequence required for binding to mitochondria (Polakis and Wilson 1985; Gelb et al. 1992), as judged from its lack of reactivity (G. Kopf, personal communication) with monoclonal antibody 4D4 which recognizes an epitope in this region (Smith

and Wilson 1991a). This likely results from an alternate splicing mechanism (Mori et al. 1993). Presumably an altered N-terminal sequence results in transport of p95 to the plasma membrane of sperm rather than targeting to mitochondria.

Despite the results of Gelb et al. (1992), it is likely that the interactions with the mitochondrial membrane involve more than the hydrophobic N-terminal segment of type I hexokinase. Certainly other regions of the molecule are in close proximity to, and likely to be interacting with, the membrane (Smith and Wilson 1991a). These interactions are thought to include both hydrophobic and electrostatic components (Felgner and Wilson 1977). Divalent cations, with Mg^{2+} probably being most physiologically relevant, markedly enhance binding of type I hexokinase to mitochondria (Rose and Warms 1967; Felgner and Wilson 1977). These are thought to bridge between negatively charged groups on the membrane and enzyme (Felgner and Wilson 1977; Aubert-Foucher et al. 1984). The charged groups on the membrane are

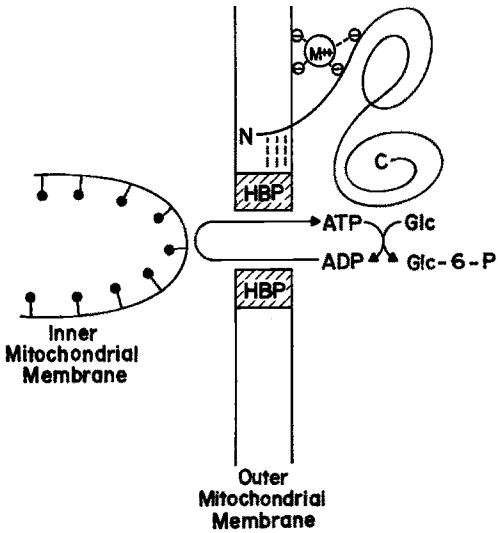

Fig. 9. Schematic representation of the interaction of the mammalian type I hexokinase with mitochondria. The enzyme is bound to the outer surface of the outer mitochondrial membrane by interactions between the N-terminal half of the enzyme and moieties on or in the membrane. These include insertion of a hydrophobic N-terminal segment of the enzyme into the core of the lipid bilayer, as well as divalent cation-mediated interactions with groups on the membrane surface. Either or both of these interactions involve an outer mitochondrial membrane protein originally isolated as the hexokinase binding protein (*HBP*) but now known to be identical with the pore forming protein (porin, VDAC) responsible for formation of the channel through which metabolites cross the outer mitochondrial membrane. This provides a topological basis for metabolic coupling between mitochondrially bound hexokinase and intramitochondrial oxidative phosphorylation. Reprinted with permission from Wilson (1988) in Microcompartmentation (D.P. Jones, ed). Copyright CRC Press, Boca Raton, Florida

presumed to be phospholipids, and binding of the enzyme is influenced by membrane phospholipid composition (Moller and Wilson 1983). A schematic representation of the mitochondrial binding of hexokinase, as presently envisaged, is shown in Fig. 9.

Based on the results of crosslinking studies, Xie and Wilson (1990) concluded that at least a portion of the rat type I hexokinase that had been bound to rat liver mitochondria existed as a tetramer, i.e., four closely associated hexokinase molecules, which were suggested to surround the pore structure. Based on the amount of hexokinase activity bound per porin molecule in rat liver mitochondria, Wicker et al. (1993) estimated four hexokinase molecules per porin and noted the agreement with the results of Xie and Wilson (1990). However, Wicker et al. assumed a specific activity of 10 U/mg for the type I hexokinase, well below the value of 60 U/mg found for the purified enzyme (Wilson 1989). If the latter specific activity is used, the calculated ratio of hexokinase to porin would be much closer to 1:1. Thus, the proposed tetrameric status of the mitochondrially bound enzyme requires additional verification. Whether this is unique to the interaction of type I hexokinase with liver mitochondria, which seems unlikely, or generally applicable to mitochondrially bound hexokinase also remains an open question, as do the possible consequences for hexokinase function.

In light of the extensive evidence for binding of type I hexokinase to mitochondria in various normal tissues, it was not surprising that Parry and Pedersen (1983) found this to also be the case with Novikoff hepatoma cells. In a subsequent study of hexokinase distribution in normal kidney, Parry and Pedersen (1984) concluded that the enzyme was primarily associated with a "light" (i.e., banding at lower density after density gradient centrifugation) population of mitochondria, in agreement with an earlier study by Wilson and Felgner (1977). However, Parry and Pedersen noted that hexokinase was not released in parallel with monoamine oxidase, an accepted outer mitochondrial marker, during digitonin fractionation. Rather, release of hexokinase appeared more similar to release of putative microsomal marker enzymes. Parry and Pedersen then speculated that hexokinase may be bound not to mitochondria per se but to actin filaments associated with the outer mitochondrial membrane; how this might be relevant to explaining correlated release of hexokinase and microsomal enzymes was not made clear. In a more extensive study with brain mitochondria, Parry and Pedersen (1990) concluded that type I hexokinase was not bound to mitochondria but rather to a "nonmitochondrial receptor" present in the

mitochondrial fraction. Pedersen and colleagues have since expanded this into a general hypothesis (Arora et al. 1992b) in which binding of hexokinase to mitochondrial or nonmitochondrial receptors is said to be correlated with transformed or normal status, respectively, of cells. We do not believe this hypothesis is well founded.

Since the study with brain mitochondria (Parry and Pedersen 1990) has been the most extensive, we use this to illustrate some of what we find troubling about the interpretations by Parry and Pedersen:

1. A brain mitochondrial preparation was fractionated by linear sucrose density gradient centrifugation. Parry and Pedersen reported that a putative "microsomal" marker, NADPH-cytochrome *c* reductase, distributed broadly throughout the gradient, including the mitochondrial region. This in itself is surprising, since microsomal and mitochondrial fractions are typically rather well resolved by such methods (e.g., Criss 1970). Furthermore, there is evidence that the outer mitochondrial membrane itself may contain NADPH-cytochrome *c* reductase, at least in liver mitochondria (Brunner and Bygrave 1969), and hence its use as a microsomal marker is ambiguous. The presence of NADPH-cytochrome *c* reductase in the outer mitochondrial membrane could explain the occurrence of significant amounts of this activity in the mitochondrial region of the gradient. It might also explain why the release of hexokinase and NADPH-cytochrome *c* reductase was similar in digitonin fractionation experiments. Moreover, the disruption of the outer mitochondrial membrane by digitonin is known to be nonuniform, i.e., even generally accepted markers are not released in parallel (e.g., Dorbani et al. 1987; BeltrandelRio and Wilson 1992a); correlation between release of enzymes from the outer mitochondrial membrane and from microsomal contaminants could merely be coincidental.

2. Parry and Pedersen subjected brain mitochondria to loading with barium phosphate, creating a second, more dense "submaximally loaded" mitochondrial population that was resolved from nonloaded mitochondria by sucrose density gradient centrifugation. Parry and Pedersen (1990) noted that "although some hexokinase ... moves to the more dense region of the gradient, it does not peak with the mitochondrial marker enzyme" (succinate dehydrogenase). Why, if hexokinase is not associated with loaded mitochondria, should it move to a "more dense region" at all? In fact, this result can readily be explained by the well-known heterogeneity of the mitochondrial population in brain (Neidle et al. 1969; Blokhuis and Veldstra 1970; Wilson 1972a); as a result of this heterogeneity, lack of congruence in distribution of various mitochondrial enzymes, including hexokinase, on density gra-

dients is indeed the norm. Parry and Pedersen (their Fig. 1) themselves noted a lack of congruence between the distribution of two mitochondrial markers, monoamine oxidase and succinate-cytochrome reductase, but this did not lead them to conclude that one of these was improperly identified as a mitochondrial component.

The results of the "submaximal loading" experiment can be taken to indicate that hexokinase-bearing mitochondria were less susceptible to barium phosphate loading; in fact, heterogeneity among brain mitochondria with respect to uptake of heavy metal ions has been noted previously (Kerpel-Fronius and Hajós 1971; Hajós and Kerpel-Fronius 1973), with hexokinase-rich nerve terminal mitochondria (Wilson 1972a; Kao-Jen and Wilson 1980) being, for reasons that have not been defined, particularly poor at succinate-dependent (as used by Parry and Pedersen 1990) ion uptake.

In their earlier study, Parry and Pedersen (1983) performed a similar "barium phosphate loading" experiment with tumor mitochondria, concluding that hexokinase migrated with mitochondrial markers in the loaded mitochondria. It is interesting to compare these results and their interpretation with the brain mitochondrial study. As with brain (Parry and Pedersen 1990), Parry and Pedersen (1983) reported that NADPH-cytochrome *c* reductase activity, and quite low activity at that, was broadly distributed throughout a linear sucrose gradient; this failure to see a peak at the density expected for endoplasmic reticulum is puzzling. A "peak" coinciding with location of mitochondrial markers consisted of a *single point* above a rather uniform baseline activity seen throughout the gradient. After barium phosphate loading, this "peak" was gone and there was, if anything, the appearance of a broad peak of NADPH-cytochrome *c* reductase activity in the same region where marker enzymes revealed the position of the loaded mitochondria. While this was said by Pedersen and Parry to indicate "no obvious specific effect of loading on the distribution of the endoplasmic reticulum marker NADPH-cytochrome *c* reductase," it could just as well be interpreted (though admittedly the low activities and ill-defined distribution make this tenuous) as indicating the association of NADPH-cytochrome *c* reductase activity with tumor mitochondria. The density gradient results presented by Parry and Pedersen (1983, 1990) are ambiguous at best, and certainly do not indicate a unique association of NADPH-cytochrome *c* reductase with the endoplasmic reticulum *or* a lack of association of hexokinase with mitochondria.

3. In Table IV of their paper, Parry and Pedersen (1990) present the results of an experiment in which the density of brain mitochondria was

markedly increased by "maximal loading" with barium phosphate, followed by collection of the loaded mitochondria by centrifugation. The lack of increased specific activity of hexokinase in the mitochondrial pellet was taken to indicate a nonmitochondrial location. First of all, one wonders why the specific activity of hexokinase should not have *decreased* if mitochondria devoid of hexokinase were selectively removed from hexokinase-bearing nonmitochondrial components by the loading procedure; this was not observed. Moreover, in this same experiment, a 13-fold increase in specific activity of the outer membrane marker, monoamine oxidase, was seen, while the specific activity of the inner membrane marker, succinate-cytochrome *c* reductase, increased only twofold. Given this large discrepancy in distribution of acknowledged mitochondrial markers, the failure to see an effect on hexokinase specific activity is less impressive, particularly since the submaximal loading experiments had indicated that hexokinase-bearing mitochondria might, for whatever reason, be less susceptible to this treatment.

We believe that the results of Parry and Pedersen (1983, 1984, 1990) do not effectively challenge the extensive work by many investigators, mentioned above, that has led to the conclusion that, in brain and other normal tissues, type I hexokinase is indeed primarily associated with the outer membrane of mitochondria.

Magnani et al. (1993) have recently provided results interpreted as indicating that, in rabbit brain, "most of the enzyme is in mitochondrial form" but a "significant fraction is bound to different intracellular structures, probably microsomes." In marked contrast to previous studies (Rose and Warms 1967; Kropp and Wilson 1970; Felgner et al. 1979), Magnani et al. (1993) reported binding of hexokinase to isolated microsomes that, on a per milligram protein basis, was comparable to that seen with intact mitochondria. They also presented immunolocalization results indicating hexokinase associated with the *inner* regions of brain mitochondria, a finding in disagreement with several earlier studies. There are alternative interpretations for the results of Magnani et al. (1993), some touched on below, but it is not our intention to further extend this critique. The interested reader will undoubtedly examine the experimental evidence and form their own opinion.

Mitochondrially bound hexokinase from rat brain can be almost totally released by incubation of the mitochondria with glucose 6-phosphate (Wilson 1989; Kabir and Wilson 1993); the possible physiological significance of this property is discussed below. In contrast, incubation of human brain mitochondria with glucose 6-phosphate results in release of only about 20% of the hexokinase, with intermedi-

ate amounts of bound hexokinase released by incubation of brain mitochondria from other species (bovine, ovine, porcine, guinea pig, rabbit) with glucose 6-phosphate (Kabir and Wilson 1993). Similar results were reported by Parry and Pedersen (1983) for hexokinase of Novikoff ascites tumor mitochondria, with about 15% of the activity resisting solubilization by glucose 6-phosphate. Hexokinase not released by glucose 6-phosphate *can* be eluted with 0.5 *M* KSCN; comparison of the glucose 6-phosphate-solubilized and KSCN-solubilized hexokinases from bovine brain mitochondria did not disclose discernible differences between the enzymes, leading Kabir and Wilson (1993) to suggest that the difference must lie with the mitochondrial binding sites themselves, i.e., there are types of binding sites on brain – and presumably tumor (Parry and Pedersen 1983) – mitochondria, with the relative proportions varying between species. Currently available results (Kabir and Wilson 1993, and unpublished work) suggest that these two types of sites coexist on a single mitochondrion, i.e., they cannot be explained based on mitochondrial heterogeneity (Neidle et al. 1969; Blokhuis and Veldstra 1970; Wilson 1972a). The nature of the differences between these sites that might lead to the observed difference in glucose 6-phosphate-induced release, and their possible physiological significance, remain unknown or the subject of speculation (Kabir and Wilson 1993).

Like type I hexokinase, the type II isozyme has been reported to bind to mitochondria in various mammalian tissues (e.g., Katzen et al. 1970; Salotra and Singh 1982; Lawrence and Trayer 1985) and tumor cells (e.g., Kosow and Rose 1968; Kurokawa et al. 1982; Vischer et al. 1987; Radojkovic and Ureta 1987). At least 80% of both isozymes could be released by incubation of tumor mitochondria with glucose 6-phosphate (Kurokawa et al. 1982; Parry and Pedersen 1983; Radojkovic and Ureta 1987). The type I and II isozymes compete with each other for binding, and treatment of either with chymotrypsin results in loss of binding ability (Kurokawa et al. 1982). It is therefore reasonable to suggest that the type I and II isozymes bind in a similar manner (Fig. 9), although it does not appear that experiments directly demonstrating interaction of the type II isozyme with membranes containing purified porin have been done, as they have for the type I isozyme (Felgner et al. 1979; Fiek et al. 1982; Lindén et al. 1982). The type II isozyme does possess a moderately hydrophobic N-terminal sequence which might interact in the same manner as the corresponding sequence of type I hexokinase (Xie and Wilson 1988), but it does not include a residue equivalent to Lys-15 of type I hexokinase, suggested by De Pinto et al.

(1993) to interact with Glu-72 of porin (see above). Perhaps these differences contribute to an apparent diminished affinity of the type II isozyme for binding to mitochondria (Kurokawa et al. 1982; Imai et al. 1988). As with the type I isozyme, discussed above, loss of binding ability can occur during purification of the type II isozyme (Zelenina et al. 1991); this presumably results from modification of the hydrophobic N-terminal segment by endogenous proteases, but this has not been directly demonstrated.

Although there are reports to the contrary (Gellerich and Augustin 1979; Denis-Pouxviel et al. 1987), most studies have indicated that the type III isozyme is not associated with mitochondria (Katzen et al. 1970; Salotra and Singh 1982; Radojkovic and Ureta 1987) but is found in the soluble fraction of cellular extracts (however, see further comments below). In at least one instance, a mitochondrially bound isozyme tentatively identified as type III hexokinase (Parry and Pedersen 1983) was subsequently shown (Radojkovic and Ureta 1987) to be the type II isozyme. Similarly, it seems to be generally accepted (Iynedjian 1993) that hepatic type IV hexokinase is located in the cytosol (but see below); both histochemical (Lawrence et al. 1984) and immunohistochemical (Kirchner et al. 1993) studies are consistent with this, although a predominantly nuclear location has also been claimed (Miwa et al. 1990). An inability of type III and type IV hexokinase to bind to mitochondria, at least in a manner similar to that with the type I and II isozymes, is readily explicable based on examination of amino acid sequences of these isozymes (Fig. 2). Both type III and type IV hexokinase lack the highly hydrophobic N-terminal character that is critical for binding to mitochondria (Polakis and Wilson 1985; Gelb et al. 1992).

7.1.1.2 Functional Significance of the Binding of Hexokinase to Mitochondria

This aspect of hexokinase has been discussed in previous articles (Wilson 1985, 1988); these may be consulted for more extensive comments about earlier work in the area, which are only briefly summarized in the present context.

The association of an ATP-requiring, ADP-producing enzyme like hexokinase with an ADP-requiring, ATP-producing organelle like the mitochondrion understandably led to speculation about the possibility that such physical proximity might lead to close metabolic interaction (Rose and Warms 1967). The concept that hexokinase might have "privileged access" to ATP produced by intramitochondrial processes, par-

ticularly oxidative phosphorylation, and that ADP produced by mitochondrially bound hexokinase might be efficiently redirected back for rephosphorylation, developed early on and was supported by observations from a number of laboratories (see Wilson 1985), perhaps most notably that of Bessman (Bessman and Geiger 1980; Viitanen et al. 1984). This general concept has been supported by more recent work with tumor (Denis-Pouxviel et al. 1987; Arora and Pedersen 1988; Gauthier et al. 1989, 1990) and rat brain (BeltrandelRio and Wilson 1991, 1992a,b) mitochondria, and extensively discussed by several investigators (Nicolay et al. 1990; Brdiczka 1991). At relatively high, and probably unphysiological, concentrations of ADP, adenylate kinase (located in the intermembranal space) can generate ATP as a substrate for mitochondrially bound hexokinase (Nelson and Kabir 1985; BeltrandelRio and Wilson 1991). However, at more physiological concentrations of ADP (approximately 30 μM), it seems clear that oxidative phosphorylation is the primary source of substrate ATP (Arora and Pedersen 1988; Gauthier et al. 1989; BeltrandelRio and Wilson 1991).

BeltrandelRio and Wilson (1991, 1992a,b), working with rat brain mitochondria, concluded that, *during active oxidative phosphorylation*, mitochondrially bound hexokinase used intramitochondrial ATP exclusively as a substrate. An example of the experimental results leading to this view is shown in Fig. 10. Although the *initial* rate of glucose phosphorylation was dependent on extramitochondrial ATP levels present at the time of initiating oxidative phosphorylation (by addition of ADP), the rate of glucose phosphorylation ultimately approached a steady-state value that was *independent* of the extramitochondrial ATP concentration remaining; this steady-state rate was approximately half the rate of glucose phosphorylation seen with saturating levels of extramitochondrial ATP in the absence of oxidative phosphorylation. The steady-state rate of glucose phosphorylation by hexokinase bound to phosphorylating mitochondria was correlated with the rate of oxidative phosphorylation when the latter was manipulated by variation in the substrate (pyruvate/malate or P_i) supply. The interpretation of these and other results was that initiation of oxidative phosphorylation resulted in some undefined but presumably structural change in the hexokinase-mitochondrial interaction that led to exclusive focus on intramitochondrial ATP as substrate. Addition of creatine phosphate to actively phosphorylating mitochondria, permitting generation of additional ATP via the mitochondrial creatine kinase reaction, resulted in a doubling of the rate of ATP production but only a marginal (approximately 10% increase)

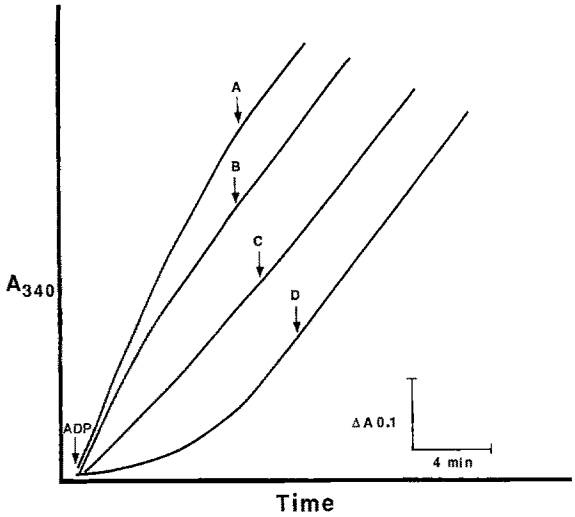

Fig. 10. Phosphorylation of glucose by hexokinase bound to rat brain mitochondria, with ATP generated by oxidative phosphorylation in the presence of increasing extramitochondrial concentrations of ATP. ATP production was initiated by addition of ADP at time zero. Also present in the reaction medium at time zero were 1.1 m*M*, 0.66 m*M*, 0.22 m*M*, or 0 m*M* ATP for *curves* A–D, respectively. Glucose phosphorylation was monitored spectrophotometrically by coupling glucose 6-phosphate formation to NADPH production, observed at 340 nm. Although the initial rate of glucose phosphorylation was markedly dependent on extramitochondrial ATP concentrations, all reactions approached a common steady state rate despite the fact that there were marked differences in the amount of ATP remaining in the extramitochondrial medium, e.g., *curve A* (initial ATP concentration of 1.1 m*M*), more than 90% of the originally added ATP remained. Reprinted from BeltrandelRio and Wilson (1992b) with permission of Academic Press.

in the rate of glucose phosphorylation; this suggested that the mitochondrial hexokinase was almost totally dependent on an ATP compartment uniquely associated with oxidative phosphorylation. The physiological significance of this dependence was suggested (BeltrandelRio and Wilson 1992a) to be coordination of glycolytic (for which hexokinase represents the initial step) and oxidative phases of glucose metabolism, avoiding formation of lactate which is thought to be neurotoxic (Marie and Bralet 1991).

In agreement with the results of BeltrandelRio and Wilson (1991), Kabir and Nelson (1991) reported that the steady-state rate of glucose phosphorylation with saturating levels of extramitochondrial ATP as substrate for hexokinase, bound to *nonphosphorylating* rat brain mitochondria, was two- to threefold higher than the maximum rate seen with ATP supplied by oxidative phosphorylation. These authors did not

examine the relationship between extramitochondrial ATP levels and the rate of glucose phosphorylation by hexokinase bound to actively phosphorylating mitochondria (e.g., experiment depicted in Fig. 10). In contrast to the results of BeltrandelRio and Wilson (1992b), Kabir and Nelson (1991) found that the rate of glucose phosphorylation with ATP supplied by the creatine kinase reaction was considerably greater than that with ATP supplied by oxidative phosphorylation. The explanation for these divergent findings, under similar reaction conditions, remains to be determined.

With excess (and nonmitochondrially bound) yeast hexokinase added as a scavenger of extramitochondrial ATP, addition of KCN or several other inhibitors of electron transport, adenine nucleotide translocase, or oxidative phosphorylation to phosphorylating rat brain mitochondria resulted in a burst of NADPH formation in an assay system in which glucose 6-phosphate formation was coupled to NADPH production via the glucose 6-phosphate dehydrogenase reaction (BeltrandelRio and Wilson 1991). This was interpreted as indicating release of ATP from intramitochondrial compartments, with the increment in NADPH taken as a measure of compartmented ATP. This interpretation is now known to be incorrect, and the KCN-induced increment in NADPH formation to be an artifact associated with the assay conditions (Laterveer et al. 1993); the source of this artifact remains undetermined. While this obviously requires reevaluation of this aspect of the work by BeltrandelRio and Wilson (1991, 1992a,b), it should be noted that their major conclusion – that glucose phosphorylation by mitochondrial hexokinase is closely coupled to intramitochondrial ATP production by oxidative phosphorylation – rested on comparison of steady-state rates for these processes, and hence remains tenable. Nonetheless, it is clear that additional work, with alternative approaches, is required to firmly establish the suggested coupling of oxidative phosphorylation and glucose phosphorylation, and to understand the physical basis by which it might occur.

Pedersen and his colleagues (Pedersen 1978; Bustamante and Pedersen 1977; Bustamante et al. 1981; Arora and Pedersen 1988) have noted that high levels of mitochondrially bound hexokinase are found in several rapidly growing tumor cell lines. "Preferred access" of the mitochondrial hexokinase to ATP generated by oxidative phosphorylation, together with decreased sensitivity of the mitochondrially bound enzyme to inhibition by glucose 6-phosphate (discussed below), have been suggested (Arora and Pedersen 1988) to account for the high glycolytic rate characteristic of such tumors. The situation is undoubtedly

more complex, however. Although 75% of the total hexokinase was reported to be mitochondrially bound in HT29 adenocarcinoma cells grown under conditions which led to either poorly differentiated, highly glycolytic or highly differentiated, less glycolytic states, Murat and coworkers (Denis-Pouxviel et al. 1987; Gauthier et al. 1989, 1990) found *decreased* coupling of mitochondrial hexokinase to oxidative phosphorylation in the highly glycolytic, poorly differentiated tumor cells while hexokinase bound to mitochondria from the highly differentiated, less glycolytic cells was said to be "almost totally dependent on the ATP generated by oxidative phosphorylation" (Gauthier et al. 1989); as noted above, the latter also seems to be the case with mitochondrial hexokinase from rat brain, surely a highly differentiated tissue.

7.1.1.3 Changes in Intracellular Distribution of Hexokinase as a Regulatory Mechanism

Early studies demonstrated that binding of hexokinase to mitochondria from bovine brain or sarcoma 37 ascites tumor cells (Rose and Warms 1967) or from rat brain (Wilson 1968) was reversed by glucose 6-phosphate, and that, as with inhibition by this ligand (see above), the solubilizing effect of glucose 6-phosphate was antagonized by P_i. These observations led to a proposal that the intracellular distribution of hexokinase between mitochondrially bound and dissociated forms, governed by intracellular levels of glucose 6-phosphate and P_i, might be a factor in regulation of in vivo hexokinase activity (Wilson 1968). The bound form was originally envisaged to be more active due to effects of binding on kinetic parameters of the enzyme, including a several-fold increase in the K_i for, and hence diminished sensitivity to inhibition by, glucose 6-phosphate; subsequent discussions of this hypothesis (Wilson 1985, 1988) also included consideration of the "preferred access" to intramitochondrially generated ATP that binding to mitochondria might provide. At the time of the original proposal (Wilson 1968), the distribution of hexokinase was viewed as an "either/or" proposition, i.e., either it was bound or it wasn't. As more was learned about the interaction between enzyme and mitochondria, this aspect too was modified to include provision for a form of the enzyme that had bound, and been inhibited by, glucose 6-phosphate but had not yet dissociated from the mitochondrion (Wilson 1985, 1988). The adjectival term "ambiquitous" was suggested (Wilson 1978b) to describe enzymes, like hexokinase, that might exhibit functionally significant changes in intracellular distribution in response to altered metabolic status. Results indicating ambiq-

uitous behavior of hexokinase in response to increased or decreased glycolytic activity in rat brain have been discussed previously (Wilson 1988). More recent work by Lynch et al. (1991) is also consistent with ambiquitous behavior of hexokinase in cultured rat astrocytes.

In the present context, it is interesting to note the work of Laursen et al. (1990) who examined hexokinase distribution in brains of mice differing in their response to withdrawal after chronic exposure to alcohol. Acute administration of alcohol to mice from a strain prone to seizures during withdrawal resulted in increased hexokinase in the soluble fraction of brain homogenates, while *decreased* soluble hexokinase was found after acute alcohol dosing of mice from a strain resistant to seizures during withdrawal. In agreement with previous studies in rat, chick, and mouse brain (see Wilson 1988), ischemia induced a rapid shift of hexokinase activity toward the bound form in brain from both strains. Thus, it would appear that the seizure-prone and seizure-resistant strains differ in the effect of alcohol on hexokinase distribution, but not in their response to the effect of ischemia on that distribution. From remarks above it is apparent that alcohol-induced changes in hexokinase distribution might have implications with respect to cerebral glucose metabolism, but how this might be – and indeed, whether it really is – related to induction of seizure activity remains unclear.

As noted above, the original suggestion that changes in intracellular distribution of rat brain hexokinase might be involved in regulation of hexokinase activity in vivo (Wilson 1968) has been revised in light of new information about the hexokinase-mitochondrial interaction. Originally envisaged as a primary regulatory mechanism, redistribution of the enzyme has come to be viewed as a secondary factor, operative only with more extreme perturbations of cerebral energy metabolism (BeltrandelRio and Wilson 1992a). Under normal conditions, in which moderate fluctuations in glycolytic rate in response to altered neurophysiological function occur, alteration of the activity of the mitochondrially bound hexokinase in response to changes in rate of mitochondrial oxidative phosphorylation has been suggested to be of primary importance. Implicit in this view is the assumption that hexokinase not bound to mitochondria makes only a minor contribution to the total rate of glucose phosphorylation. Lowry and Passonneau (1964) estimated that only about 10% of the full catalytic potential of hexokinase would be necessary to sustain normal rates of cerebral glucose metabolism, indicating that the enzyme is highly regulated in vivo; given the increased sensitivity of the dissociated form to inhibition by glucose

6-phosphate, it is perhaps not unreasonable to assume that the mitochondrially bound form plays the major role in cerebral glucose phosphorylation. Nonetheless, this must be recognized as the oversimplification that it is, and it is not extrapolatable to tissues in which, unlike brain, a much lower fraction of the hexokinase activity appears to exist in mitochondrially bound form.

The hypothesis that changes in intracellular distribution of hexokinase might be involved in regulation of activity in vivo was largely developed based on work with rat brain. Recent recognition that the extent to which mitochondrial hexokinase is sensitive to release by glucose 6-phosphate varies markedly among species (Kabir and Wilson 1993), with rat being one extreme, must be taken into account when considering the applicability of this hypothesis to other species. It remains to be determined whether the mitochondrially bound forms differing in sensitivity to solubilization by glucose 6-phosphate also differ in the nature of coupling to intramitochondrial ATP generating processes.

It is surely apparent that factors governing the in vivo activity of hexokinase are more complex than might have been envisaged even a few years ago. Much remains to be added to our understanding of the metabolic roles of the mitochondrially bound and dissociated forms of the enzyme, in brain as well as in other tissues.

7.1.2 Binding of Hexokinase to Other Cellular Membranes

Although both type I and type II hexokinase can bind to mitochondria, significant amounts of these isozymes have been found in other fractions obtained by centrifugal fractionation techniques (Katzen et al. 1970). It seems likely that some of this might simply represent contamination of other particulate fractions with mitochondria or with disrupted outer mitochondrial membrane fragments that have retained bound hexokinase. Nonetheless, the possibility that hexokinase may be associated with other cellular membranes must certainly be considered. As noted above, mitochondrial porin or related members of a protein family may be located in other cellular membranes (Thinnes 1992); whether this is functional in binding of hexokinase remains to be determined.

7.1.2.1 Possible Involvement in Glucose Transport Across the Plasma Membrane

As mentioned earlier, there have been suggestions that the glucokinase of yeast may be somehow involved in transport of glucose into the cell. Although this might suggest physical association of the enzyme with the

plasma membrane, there does not appear to be any direct evidence for this. The same can be said for mammalian systems, with the exception of the report by Emmelot and Bos (1966) that significant hexokinase activity – isozyme not specified but probably type I and/or type II – was associated with the plasma membrane from rat hepatoma cells but not with the plasma membrane of hepatocytes. It does not appear that this early report has been followed up, and the possible functional significance remains only speculative.

Pedley et al. (1993) have reported that stimulation of macrophages with a phorbol ester resulted in increased levels of hexokinase at the cell periphery, with decreased levels in more interior regions of the cell. This was presumably the type I isozyme since antiserum against the human type I hexokinase was used for immunofluorescent detection of the enzyme, but crossreactivity with other isozymes is a possibility that must be considered. No change in intracellular distribution of another enzyme, glucose 6-phosphate dehydrogenase, was seen under these same conditions. Cytochalasin D prevented translocation of hexokinase, implicating actin microfilaments in the process. Pedley et al. suggested that phorbol ester-stimulation resulted in enhanced association of hexokinase with actin microfilaments adjacent to the plasma membrane and that this was somehow involved in the increased glucose transport and metabolism associated with macrophage activation. However, the phorbol ester-induced increase in hexokinase at the cell periphery was, quantitatively, quite modest (the quantitative analysis shown in Fig. 5 of Pedley et al. is in striking contrast to the pronounced redistribution of fluorescence suggested by the micrograph in their Fig. 4) as were the effects on kinetics of 2-deoxyglucose uptake found by Pedley et al. (1993). Thus, the possible relationship between translocation of hexokinase and metabolic changes resulting from macrophage activation requires further definition.

Transport of glucose into most mammalian cells is mediated by one or more members of a family of glucose transporters currently known to include at least four proteins, designated GLUT1-4; other members of this family are involved in fructose transport, or glucose transport into the endoplasmic reticulum (Bell et al. 1993a; Gould and Holman 1993). GLUT1-4 differ in various properties including substrate specificity, relative affinities for glucose, and tissue distribution. Bell et al. (1993a) also noted that expression of GLUT1-4 in various tissues tends to be correlated with particular isozymes of mammalian hexokinase, suggesting the possibility of a functional physical interaction between these components, although direct evidence for this is lacking. GLUT2

is, like type IV hexokinase, expressed at high levels in liver and pancreatic β-cells; however, GLUT2 is also expressed in other tissues, and the expression of these two proteins is not strictly correlated in liver (Tiedge and Lenzen 1993). Interestingly, GLUT2 has, like glucokinase, a relatively low affinity for glucose and this may be related to function as a "glucose sensor," responsive to altered concentrations of plasma glucose (see below). GLUT4 is an intriguing protein that could be said to be ambiquitous, i.e., its distribution between the plasma membrane and intracellular vesicular elements is affected by hormonal status, with increased amounts found in the plasma membrane in response to elevated insulin (Jhun et al. 1992). Like GLUT4, type II hexokinase is predominantly associated with insulin sensitive tissues (Katzen 1967). In rat adipose tissue, expression of mRNAs for type II hexokinase and GLUT4 is coordinated and responsive to insulin levels (Postic et al. 1993; Burcelin et al. 1993). However, the situation in heart *and* skeletal muscle may be somewhat different; for example, effects of streptozotocin-induced diabetes on GLUT4 and type II hexokinase mRNAs in muscle and heart were much less pronounced than in adipose tissue (Burcelin et al. 1993) and hyperinsulinemia increased both mRNAs in adipose tissue but only the mRNA for type II hexokinase in skeletal muscle (Postic et al. 1993).

Lachaal and Jung (1993) have reported that a glucokinase from *Bacillus stearothermophilus* binds to human GLUT1 reconstituted into lipid vesicles. The relevance of this as a model for possible interactions between a mammalian hexokinase and the glucose transporter is unclear, as pointed out by Lachaal and Jung themselves. Whether the *B. stearothermophilis* enzyme, with molecular weight of about 30 kDa, bears any significant resemblance to mammalian hexokinases is unknown but it seems unlikely that any structural feature required for binding of the mammalian enzyme to GLUT1 would be conserved in the much smaller bacterial enzyme. Moreover, the binding was optimal at pH 4, and was enhanced by quite unphysiological concentrations of ADP and glucose 6-phosphate, effective concentrations of these ligands being in the 5–100 m*M* range. Although a modest (approximately 50%) increase in glucokinase activity was reported to result from interaction with the glucose transporter, there was no effect on glucose transport activity.

7.1.2.2 Association of Type III Hexokinase with the Nuclear Periphery

As discussed above, the type III isozyme has generally been found in the soluble fraction of tissue homogenates, suggesting a cytoplasmic location for this isozyme. Surprisingly, immunolocalization studies using a monoclonal antibody reactive with the type III isozyme showed prominent staining at the periphery of nuclei in specific cell types in various rat tissues, including kidney, liver, spleen, lung and brain (Preller and Wilson 1992). Since no hexokinase activity was associated with nuclei isolated from kidney or liver, it was concluded that the interaction must be quite weak, being readily disrupted during the homogenization and fractionation procedure.

The study by Preller and Wilson (1992) also emphasizes the likely importance of distinct intracellular locations in the function of the hexokinase isozymes. Thus, ependymal and choroid plexus cells displayed intense staining for type III hexokinase at the nuclear periphery *and* for the type I isozyme in cytoplasmic areas of these same cells. Such compartmentation can hardly be considered irrelevant, and suggests that glucose 6-phosphate produced by the type I and type III isozymes may be directed to different metabolic fates, determined by the site of its formation.

7.2 Regulation of Mammalian Hexokinase Activity

Hexokinase represents the initiating step in metabolism of glucose by most metabolic pathways (e.g., glycolysis, hexose monophosphate shunt, glycogen synthesis), and enzymes in such position frequently play a major role in governing flux through the pathway. This may be expected to be generally true for hexokinase, and it is certainly true in mammalian systems. As with other aspects of hexokinase, discussed above, there is rather limited information that bears directly on the regulation of hexokinase in nonmammalian systems. Thus, we again find ourselves focusing primarily on the mammalian isozymes.

7.2.1 Short-Term: Regulation of Hexokinase Activity in Response to Transient Changes in Metabolic Status

It should be evident from the discussion above that there are several factors likely to be involved in regulation of hexokinase activity in response to altered metabolic status. Feedback inhibition by the

product, glucose 6-phosphate, is generally accepted as a major factor governing the activity of the 100-kDa isozymes, and obviously this could also play a role in non-mammalian organisms, such as *S. mansoni*, which possess hexokinases sensitive to this ligand. Differences between the mammalian isozymes in their sensitivity to (K_i for) glucose 6-phosphate, particularly the diminished sensitivity of the type III isozyme, could certainly be of significance. Antagonism (type I isozyme) or supplementation (type II and type III isozymes) of this effect by P_i is likely to be a modulatory influence that adapts the isozymes for distinct metabolic roles (Wilson 1985).

Glucose 1,6-bisphosphate is also a rather potent inhibitor of the mammalian isozymes (Crane and Sols 1954; Rose et al. 1974), and the potential importance of this in regulation of in vivo hexokinase activity has been particularly emphasized by Beitner and her colleagues (Beitner 1979, 1993; Beitner et al. 1979, 1982). Substrate (glucose) inhibition seen with the type III isozyme is another property that could be of importance in regulation of the enzyme in vivo but a specific metabolic scenario in which this would come into play has not yet been proposed. Superimposed on the action of effector ligands may be additional factors that result from association of hexokinase with structural elements such as mitochondria, plasma membrane, cytoskeleton, or nucleus, as discussed above.

7.2.2 Long-Term: Changes in Hexokinase Activity During Development or in Response to Chronic Alteration in Metabolic Status

In concert, various factors mentioned above provide a basis for regulating *existing* levels of hexokinase, adjusting the rate of glucose phosphorylation to meet rather rapid fluctuations in metabolic demand. In the present section, we focus on changes in the levels of the enzyme itself in the course of development or in response to various physiological perturbations.

Changes in hexokinase levels during development have been particularly well studied in brain, where the type I isozyme represents by far the major form (Katzen and Schimke 1965; Grossbard and Schimke 1966; Katzen 1967). In the rat, hexokinase levels increase several-fold within the first 3 weeks postnatally, with increases in activity of "particulate" activity being most marked (Wilson 1972b; Kellogg et al. 1974; MacDonnell and Greengard 1974; Land et al. 1977; Hothersall et al. 1979; Booth et al. 1980; Leong and Clark 1984); the latter includes enzyme bound to free mitochondria as well as hexokinase – much of it

bound to mitochondria (Wilson 1972a) – entrapped in nerve endings. The exact timing and extent of the increase vary in different brain regions (Kellogg et al. 1974; Leong and Clark 1984), being correlated with differences in the timing of development in these regions. Similar increases in cerebral hexokinase levels occur *prenatally* in the guinea pig, reflecting the earlier development of the brain in this precocial species (Booth et al. 1980). Hothersall et al. (1979) noted a correlation between developmental increases in hexokinase and the rate of glucose phosphorylation in slices prepared from brains of rats at different ages.

Developmental increases in other glycolytic enzymes also occur; in general, these are not closely correlated with the increase in hexokinase activity (Wilson 1972b; MacDonnell and Greengard 1974), although the degree of correlation depends on the particular brain region under study (Leong and Clark 1984). In view of the close relationship between the type I isozyme and mitochondria in brain (discussed above), it is of particular interest that developmental increases in hexokinase correlated with increases in cytochrome oxidase and adenylate kinase, two enzymes closely associated with mitochondrial function. As stated more than 20 years ago (Wilson 1972b), "this latter correlation argues strongly for some type of intimate relationship between the mitochondria and hexokinase and also provides support for the view that the association of hexokinase with the mitochondria is an in vivo reality."

It is also noteworthy that a similar close correlation of hexokinases with mitochondrial enzymes – and an inverse relationship between levels of hexokinase and levels of other glycolytic enzymes – has been recognized in metabolically and functionally distinct types of muscle fibers (Staudte and Pette 1972; Pette 1986; Pette and Staron 1990). High levels of hexokinase, but low levels of other glycolytic enzymes, are found in "type I" fibers, with the reverse being the case in "type II" fibers. Chronic stimulation of rabbit tibialis anterior muscle resulted in correlated increase in mitochondrial volume, and increases in hexokinase activity in "constant proportion" to other mitochondrial enzyme activities (Pette 1986). Pette (1986) interpreted the latter observation to indicate "that glucose phosphorylation is preferentially linked to ATP from oxidative phosphorylation", a concept discussed in detail above and consistent with the results of Viitanen et al. (1984).

Developmental increases and regional variations in hexokinase *activity* in neural tissues of the rat are correlated with the intensity of immunofluorescent staining, the latter providing a measure of the amount of the enzyme protein itself (Wilkin and Wilson 1977; Simurda and Wilson 1980; Gutekunst and Wilson 1981). This is not unexpected since

there are no known posttranslational modifications that affect the specific activity of type I hexokinase, and hence changes in activity are most likely attributable to changes in the amount of the type I isozyme present. Whether the levels of type I hexokinase are determined at the transcriptional level or by posttranscriptional events is not clear. The only relevant study with the type I isozyme that is known to this writer is the work of Griffin et al. (1992), who examined the relative levels of mRNA for type I hexokinase in developing brain and other tissues of the rat. They concluded that, in brain, the level of mRNA for type I hexokinase was relatively high prenatally, and increased to a maximum at about seven days postnatally before declining to adult levels by about 3–4 weeks postnatally. This may be compared with the rather low levels of hexokinase activity prenatally, with marked increase starting approximately 10 days postnatally to adult levels attained at about 3–4 weeks postnatally (Wilson 1972b; Kellogg et al. 1974; MacDonnell and Greengard 1974; Land et al. 1977). Based on the lack of correlation between relative levels of type I hexokinase mRNA and activities present in brain and other tissues at various times of development, Griffin et al. (1992) concluded that both transcriptional and posttranscriptional processes were involved in regulating levels of type I hexokinase during development. It should be noted that Griffin et al. (1992) determined type I hexokinase mRNA levels *ralative* to levels of mRNA for another glycolytic enzyme, phosphoglycerate kinase; steady-state mRNA levels for the latter enzyme were said to be relatively constant during development in all tissues except skeletal muscle. However, as noted earlier, marked increases in activities of several glycolytic enzymes are seen during maturation of the brain and it would be surprising if this did not include phosphoglycerate kinase, although the activity of this particular enzyme does not appear to have been measured directly in earlier developmental studies. It would also be surprising if developmental increases in other glycolytic enzymes did not involve, at least to some extent, transcriptional regulation. Thus, the observation of Griffin et al. (1992) that mRNA levels for phosphoglycerate kinase were relatively constant during development deserves further examination. Nonetheless, these results are consistent with the view that regulation at the trancriptional level is involved in determining developmental changes in levels of type I hexokinase; moreover, they provide a further indication that expression of type I hexokinase is not strictly correlated with that of other glycolytic enzymes, in this case, with phosphoglycerate kinase.

It was mentioned earlier that the relationship between the various types of glucose transporters and isozymes of hexokinase is of consid-

erable interest (Bell et al. 1993a). In the present context, it is pertinent to note the recent study by Vannucci (1994) in which levels of GLUT1 and GLUT3 glucose transporters were determined (by Western blotting) during development of rat brain. Although Vannucci did not explicitly compare her results with earlier studies on hexokinase, it is clear that developmental increases in levels of GLUT3 and hexokinase occur with similar timing; for example, in cerebral cortex, both GLUT3 (Vannucci 1994) and hexokinase (Kellogg et al. 1974) approximately double during the first postnatal week, with further increase to adult levels attained at 3–4 weeks postnatally. In contrast, there was relatively little change in GLUT1 during the first 2–3 weeks postnatally, followed by a sharp rise to adult levels at about 4 weeks postnatally. Vannucci suggested that GLUT1 is associated with vascular and glial elements in the brain, while GLUT3 is primarily neuronal. Increases in GLUT3 were thought to be "more tightly linked to the level of maturity and functional activity of neuronal populations" and to directly reflect rates of glucose utilization in various brain regions; as discussed below, this is also thought to be the case with hexokinase activity (Burgess and Wilson 1991). While it is likely that GLUT1 and GLUT3 play rather distinct roles in cerebral metabolism, it is also likely that they are both linked to glucose phosphorylation by type I hexokinase which, as noted earlier, is by far the predominant isozyme found in brain.

The type I isozyme is also predominant in glial tumors that have been examined, although substantial levels of the type II isozyme may also be present (Oude Weernink et al. 1991). While GLUT1 may be the predominant transporter in normal glia (Vannucci 1994), GLUT3 seems to be the predominant form present in glial tumors (Nagamatsu et al. 1993). Nagamatsu et al. also detected the GLUT1 and GLUT4 transporters in astrocytic tumors, although with much lower frequency than the GLUT3 transporter which was present in every astrocytoma examined.

As with the type I hexokinase itself (see Wilson 1985), the levels of mRNA for this isozyme vary markedly in different tissues (Griffin et al. 1992). It is interesting to note that there may be variation with species in the expression of this isozyme in particular tissues, the prominence of the type I isozyme in human skeletal muscle, in contrast to the predominance of type II hexokinase in skeletal muscle from other species (references cited in Wilson 1985), being one example. Wen and Bekhor (1993) have recently reported a difference in the expression of type I hexokinase in the lens of mice and rats which may account for the greater propensity for cataract formation in rats compared to mice. Wen and

Bekhor observed that, relative to levels of mRNA for aldose reductase, expression of mRNA for type I hexokinase was markedly enhanced in mouse lens. These authors suggested that expression of higher levels of hexokinase in mouse lens might favor metabolism of glucose via the glycolytic pathway, and away from the aldose reductase-requiring polyol pathway that leads to accumulation of carbohydrates associated with cataract formation.

Yokomori et al. (1992) reported that levels of mRNA for type I hexokinase were increased in response to treatment of cultured rat thyroid FRTL5 cells with thyroid stimulating hormone (TSH). That this was due to transcriptional regulation was shown by the observations that TSH had no effect on stability of the mRNA and that TSH increased the synthesis of the type I mRNA in nuclear run-on assays. Similar results were obtained by treatment of the cells with dibutyryl cyclic AMP or with forskolin, implicating 3′,5′-cyclic AMP in regulation of transcription of the type I hexokinase gene.

Thyroid hormone is known to have a major influence on development of the brain (Balázs et al. 1975); postnatal increases in hexokinase are delayed in hypothyroid rats and accelerated in hyperthyroid animals (Gutekunst and Wilson 1981). Although the effect of thyroid hormone has not been demonstrated at the transcriptional level, this seems likely to be involved.

Ketone bodies represent important substrates for the developing brain (Sokoloff et al. 1977), and it was conceivable that increased availability of ketone bodies, as an alternative to glucose, might influence developmental changes in hexokinase activities. However, although prenatally induced and postnatally maintained ketosis did influence levels of β-hydroxybutyrate dehydrogenase in developing rat brain, this had no effect on timing or extent of the postnatal increases in hexokinase activity (Sherman and Wilson 1978).

Using quantitative immunofluorescence techniques, the levels of type I hexokinase in various regions of rat brain have been correlated with basal rates of glucose utilization in these regions (Turek et al. 1986; Burgess and Wilson 1991). In the latter study, however, certain regions – referred to as "group II" regions – were found to contain hexokinase levels higher than what might be expected based on basal glucose utilization rates. It was suggested that group II regions might be adapted to sustain function-dependent increases in glucose utilization that, relative to basal rates, were considerably greater than those seen in "group I" regions, the latter being regions in which hexokinase levels and basal metabolic rates *were* correlated. In other words, certain regions of the

brain might be adapted to sustain an exceptionally large range of changes in glucose utilization rates. That hexokinase levels are generally correlated with energy demands was also suggested by the work of Dietrich et al. (1981) in which removal of specific whiskers resulted in decreased hexokinase activity (and activities of other enzymes associated with energy metabolism) in somatosensory cortical "barrels" (indirectly) innervated by the affected whiskers; such treatment had previously been demonstrated to reduce the rate of glucose utilization in the affected "whisker barrel" (Durham and Woolsey 1977). Hexokinase activity, determined histochemically in various discrete brain regions, has been reported to be influenced by a number of physiological perturbations, including water deprivation (Krukoff et al. 1986; Krukoff and Vincent 1989), hypertension (Turton et al. 1986; Krukoff 1988; Simon et al. 1989; Krukoff and Weigel 1989), streptozotocin-induced diabetes (Krukoff and Patel 1990), and surgically-induced heart failure (Patel et al. 1993); such changes have been taken to reflect persistently altered metabolic activity in these regions in response to the perturbation.

Levels of type II hexokinase in "fast-twitch" skeletal muscle also are responsive to chronic changes in function-related energy demand. Although the magnitude of the effect varies somewhat in different species (Simoneau and Pette 1988), chronic stimulation results in increased activity of mitochondrial enzymes *and* hexokinase, and decreased activities of other glycolytic enzymes (Pette 1986; Simoneau and Pette 1988). A stimulation-induced increase of up to 14-fold in the hexokinase activity in rat tibialis anterior muscle has been shown to be correlated with an increase in the actual amount of type II isozyme present, resulting from an increased rate of synthesis (Weber and Pette 1988), i.e., this is not simply activation of preexisting enzyme. Cessation of stimulation resulted in a normalization of hexokinase activity, which was associated with a marked decrease in the rate of synthesis of the type II isozyme (Weber and Pette 1990a). The vast majority of the enzyme was associated with the particulate fraction, presumably mitochondria, and increased levels of mitochondrially bound hexokinase were suggested to enhance glucose phosphorylation through coupling to intramitochondrial ATP production (Weber and Pette 1990b). The increase in hexokinase activity in response to chronic stimulation was transient – maximal at about 2–3 weeks, with subsequent rapid decline, under the conditions used by Weber and Pette (1988, 1990a,b). It was suggested that this reflected a transition from a primarily carbohydrate-based metabolism to one in which oxidative metabolism of fatty acids represented the major source of metabolic energy (Weber and Pette 1990b).

These activity-dependent changes in type II hexokinase activity might reasonably be expected to reflect, at least to some extent, regulation at the transcriptional level. Consistent with this, O'Doherty et al. (1994) have shown increased levels of mRNA for type II hexokinase after a single brief period of exercise, a remarkably prompt response. Moreover, Hofmann and Pette (1994) found detectable increase in mRNA for type II hexokinase after only one hour of low frequency stimulation of fast-twitch rat tibialis anterior muscle, with a 30-fold increase over mRNA levels in control muscle after only 12 h of stimulation. Increases in mRNA were followed by increased synthesis of type II hexokinase, and cessation of stimulation led to decreased mRNA and decreased rate of synthesis. However, activity of type II hexokinase itself was not directly correlated with rate of synthesis, and Hofmann and Pette noted that additional factors may be operative, for example, stabilization of the enzyme by increased intracellular glucose levels in stimulated muscle or by binding of hexokinase to mitochondria.

In view of the association, conceptually if not physically, of type II hexokinase with the GLUT4 glucose transporter (Bell et al. 1993a), it is also of interest that Hofmann and Pette (1994) examined the effect of low-frequency stimulation on the GLUT4 transporter. Within 30 min of stimulation, increased GLUT4 in the plasma membrane and decreased levels in intracellular membrane vesicles were found; this is consistent with the ambiquitous nature of GLUT4 mentioned earlier, with increased glucose transport capacity resulting from translocation of intracellular reserves of existing GLUT4 protein to the plasma membrane. More prolonged stimulation resulted in increased levels of GLUT4 mRNA – twofold increase after 5 days of stimulation – and increased synthesis and cellular levels of the protein itself. The temporal difference between increases in mRNAs for type II hexokinase and GLUT4 implies that expression of the genes for these proteins is not linked in skeletal muscle; as mentioned above, this is also the case in response to insulin (Postic et al. 1993; Burcelin et al. 1993).

Given the increased levels of type II protein (Weber and Pette 1988, 1990a,b; Hofmann and Pette 1994) and mRNA (Hofmann and Pette 1994; O'Doherty et al. 1994) that are seen in response to increased activity (stimulation), it seems paradoxical that increased levels of type II hexokinase are also seen after denervation (Weber and Pette 1990b), which would be expected to diminish activity-related energy demand. Similar comments might be made about the finding of Lacaille et al. (1990) that activity of hexokinase – isozyme not determined, but presumably type II – *increased* in hindlimb muscles of rats which had been

maintained for three weeks in a hindlimb suspension apparatus. However, as pointed out by Weber and Pette (1990b), increased glucose uptake and glycolysis have been shown to result from denervation (Shoji 1986; Davis and Karl 1988). Weber and Pette (1990b) suggest that enhanced glucose uptake common to both increased activity as well as denervation might be causally related to increased levels of type II hexokinase. In this connection, it is interesting to note that Wako et al. (1989) observed marked increase in type II hexokinase levels in liver and diaphragm of fetal rats developing under conditions of maternal hyperglycemia. This might suggest a more general role for glucose in regulating levels of the type II isozyme. Glucose has also been reported to promote binding of type II hexokinase to mitochondria (Goncharova and Zelenina 1991), which might be related to the increased particulate hexokinase activity in stimulated muscle reported by Weber and Pette (1990b).

It has long been known that the levels of type II hexokinase are regulated by insulin, with decreased type II hexokinase found in insulin-sensitive tissues of diabetic animals (Katzen 1967; Katzen et al. 1970). Frank and Fromm (1986a) reported that the rate of degradation of type II hexokinase was increased threefold in skeletal muscle of streptozotocin-induced diabetic rats, with a less marked increase in degradation rates for the general pool of soluble proteins; degradation rates for both hexokinase and soluble proteins were returned to normal by insulin treatment. These same authors (Frank and Fromm 1986b) – in contrast to their earlier report (Frank and Fromm 1982) which, surprisingly, was said (Frank and Fromm 1986b) to be based on results obtained with a single diabetic and single normal animal – reported that the rate of synthesis of type II hexokinase was decreased in skeletal muscle of diabetic rats, with a return to near normal values after insulin treatment. It would thus appear that regulation of type II hexokinase levels involves insulin-dependent effects on both synthesis as well as stability of the protein itself. Insulin-induced increase in synthesis likely reflects increased transcriptional activity leading to elevated levels of mRNA for type II hexokinase (Printz et al. 1993a; Postic et al. 1993). The isolation and characterization of the gene for type II hexokinase (Printz et al. 1993a) sets the stage for future studies on transcriptional regulation by insulin.

High levels of hexokinase activity characteristic of rapidly growing tumor cells (Bustamante and Pedersen 1977; Pedersen 1978) are mirrored by relatively (compared to normal rat tissues) high levels of mRNAs, particularly for the type II isozyme, in various tumor cell lines that have been examined (Johansson et al. 1985; Shinohara et al. 1991;

Thelen and Wilson 1991). The transition from high levels of type IV hexokinase and relatively low levels of the high K_m isozymes, which are characteristic of normal liver, to predominance of the low K_m forms in hepatic tumors has been described as a relatively late event in hepatocarcinogenesis (Klimek and Bannasch 1993). Factors regulating hexokinase expression in tumor cells do not appear to have been examined to any significant extent. The possible role of insulin in governing expression of the type II isozyme in tumors would seem to be a question of potential therapeutic importance that merits attention.

7.3 Gene Structure and Genetic Disorders

That there is a single gene for each of the isozymes, types I–III, has not been definitively shown, but available results suggest that this is the case (Povey et al. 1975; Katzen and Soderman 1988; Nishi et al. 1989; Wigley and Nakashima 1992; Daniele et al. 1992; Printz et al. 1993a). As mentioned above, the rat gene for the type II isozyme has been cloned and characterized by Printz et al. (1993a); the conservation of intron/exon structure between the regions coding for the N- and C-terminal halves of type II hexokinase and the gene encoding rat type IV hexokinase (Magnuson et al. 1989) clearly supports a close evolutionary relationship between these isozymes (Printz et al. 1993a; Kogure et al. 1993), as discussed earlier. A *minimal* length of 41 kilobases was estimated for the type II gene, which was comprised of 18 exons having an average size of approximately 150 nucleotides. Based on the close sequence similarity between the mammalian isozymes (Fig. 2), Printz et al. (1993a) predicted that this structure would be highly conserved in genes for the other mammalian isozymes; hence, characterization of these genes is likely to be a formidable task.

The gene for human type I hexokinase has been located on chromosome 10q22 (Nishi et al. 1989; Daniele et al. 1992), but expression of the enzyme may be influenced by other chromosomal locations (e.g., Magnani et al. 1983b). Considering the central importance of the type I isozyme – as, for example, virtually the sole mechanism for introduction of glucose into cerebral energy metabolism, for which glucose is the primary substrate – it can be anticipated that mutations having a major effect on catalytic function of this isozyme would be lethal. Indeed, more than 20 years ago Cohen et al. (1973) noted the virtual lack of polymorphism in hexokinase and other glycolytic enzymes of human brain and erythrocytes, and attributed this to the critical role of glycolysis in these tissues, with extremely limited tolerance for variation

that might affect catalytic function. Since specific interaction with subcellular structure seems to be intimately involved in hexokinase function, it is likely not just catalytic function and regulatory properties per se that must be maintained, but also the capability for appropriate interaction with other cellular components. Speaking in a more general manner, this concept was previously emphasized by Srere (1984) who attributed the large size of enzymes – larger than might be expected if catalytic function were the sole consideration – to the necessity for sufficient surface area to permit specific interactions that determined intracellular localization and integration into functional complexes of metabolically related enzymes.

Given the critical role for type I hexokinase in erythrocytes, it is perhaps not surprising that reported cases of type I hexokinase deficiency have primarily been associated with nonspherocytic hemolytic anemia (NSHA; Rijksen and Staal 1978, 1985; Rijksen et al. 1983; Magnani et al. 1985). However, assuming that similar deficiency in hexokinase occurred in brain (which, for obvious reasons, is not readily determined on NSHA patients), it does seem surprising that this condition is not commonly associated with neurological symptoms (Cohen et al. 1973; Rijksen et al. 1983; Magnani et al. 1985). Perhaps this reflects the high levels of type I hexokinase found in brain (Wilson and Felgner 1977), with a considerable reserve of catalytic capacity (Lowry and Passonneau 1964). In this context, it is of interest that significantly decreased hexokinase activities have been reported in fibroblasts and leukocytes from some patients afflicted with a familial form of Alzheimer's disease (Sorbi et al. 1990) and in autopsy samples of cerebral cortex from Alzheimer's patients (Iwangoff et al. 1980). These results led Sorbi et al. (1990) to suggest that a defect in hexokinase is involved for at least a subset of patients suffering from Alzheimer's disease, a disorder long associated with diminished rates of energy metabolism in certain brain regions. However, decreased activity of α-ketoglutarate dehydrogenase activity, another enzyme intimately involved in energy metabolism, has also been reported in brain from Alzheimer's patients (Mastrogiacomo et al. 1993); thus, a general effect on enzymes of energy metabolism, secondary to other derangements occurring during Alzheimer's disease, must be considered. The currently prevailing view is that the primary cause of Alzheimer's disease lies with something other than hexokinase deficiency (Mattson et al. 1993; Mullan and Crawford 1993; Selkoe 1993). It might also be anticipated that Alzheimer's patients with defects in type I hexokinase would be anemic, but that correlation has apparently not been established.

It was mentioned earlier that the ability of P_i to antagonize inhibition of type I hexokinase by glucose 6-phosphate appeared to be a rather labile property, being lost during prolonged storage of the enzyme (Mayer et al. 1966; Easterby and Qadri 1981). It is interesting that Rijksen and Staal (1978) detected this same loss of P_i sensitivity in the type I isozyme from erythrocytes of an NHSA patient. A P_i insensitive form (see further comments below) was seen as only a minor species in erythrocytes from normal subjects and was thought to be generated during senescence of the cells. Rijksen and Staal concluded that, "In this light we can regard the patient's erythrocyte hexokinase as being 'old before its time'."

Wigley and Nakashima (1992) reported differences between the restriction patterns seen on genomic Southern blots, probed with cDNA for the type III isozyme, using DNA from normal liver and AS-30D hepatoma cells. This same group (Wigley et al. 1993) also reported the occurrence of an unusual *Hinf* I fragment, seen on genomic Southern blots probed under low stringency with a type I hexokinase cDNA probe; this fragment seemed to be correlated with rate of tumor growth, not being seen on blots prepared with DNA from slowly growing hepatomas but being prominent in patterns with DNA from rapidly growing hepatoma cells. Assessment of the possible significance of these observations as they pertain to hexokinase function in tumor cells will certainly require further study.

Altered type II hexokinase activity has also been associated with various myopathies (Poulton and Nightingale 1988; Rijksen and Staal 1985, and references therein), but it does not appear that any of these can be attributed to a mutation in the type II hexokinase gene itself. Genetic polymorphism at the type III locus (Povey et al. 1975; Katzen and Soderman 1988) has not been associated with any metabolic disorders.

7.4 Multiple Forms of the Mammalian Isozymes

The primary methods used for distinguishing the mammalian isozymes have taken advantage of charge differences between the proteins, e.g., electrophoresis or ion exchange chromatography. In the original studies that disclosed the existence of the four isozymes (Katzen and Schimke 1965; Grossbard and Schimke 1966; Gonzalez et al. 1967), each of the isozymes behaved as a single homogenous species, eluting as discrete apparently symmetrical peaks from an ion exchange column

or migrating as single bands on starch gel electrophoresis. Subsequently, however, these same or similar methods have indicated that, under certain circumstances, microheterogeneity might be detected within a single isozyme population. In some cases, the "multiple forms" of a particular isozyme appear to arise by artifactual modifications during processing of the tissue, whereas in others the multiple forms apparently exist in vivo.

Katzen and Schimke (1965) noted that when homogenates of rat fat pad were prepared and electrophoresed in the absence of EDTA and mercaptoethanol, the type II band resolved into two bands of similar but distinct mobilities; it was subsequently shown that deletion of the mercaptoethanol was the critical factor (Katzen 1966). The more rapidly moving (toward the anode) component was designated II_a and had a mobility that was indistinguishable from that of the type II isozyme seen in the presence of mercaptoethanol, while the slower component was designated as II_b (Katzen 1966). In further studies, similar results were seen with type II hexokinase of rat heart, skeletal muscle, diaphragm, and small intestine (Katzen 1966; Katzen et al. 1970). Thus, this seems to be a general characteristic of the type II isozyme, and likely involves sulfhydryl groups on the enzyme in some way. Type II_a preferentially disappeared in response to starvation (Katzen and Schimke 1965) or streptozotocin-induced diabetes (the latter effect being reversed by insulin), leaving only a rather faintly staining type II_b band (Katzen 1966). Preparation and electrophoresis of extracts from starved or diabetic animals in the presence of mercaptoethanol, however, resulted in the occurrence of a single type II hexokinase band, staining with an intensity comparable to that seen with control animals, i.e., inclusion of mercaptoethanol "masked" (Katzen 1966) an apparent decrease in activity in response to fasting or diabetes. It seems likely that II_a represents the fully active type II isozyme, while II_b corresponds to an oxidized (disufide) and largely inactivated – residual activity approximately 10% of the original activity – form of the enzyme which can be reactivated in the presence of mercaptoethanol (Murakami and Rose 1974). Murakami and Rose (1974) noted that glucose protected type II hexokinase against oxidative inactivation and pointed out that reduced tissue glucose levels resulting from starvation or diabetes might therefore lead to increased accumulation of the II_b form. The latter was considered more susceptible to proteolytic degradation and hence the conversion of the type II (II_a) isozyme to the II_b form would lead to increased degradation rates, and ultimately decreased levels of type II hexokinase during starvation or diabetes (Frank and Fromm 1986a). However, as

noted earlier, insulin-dependent modulation of the rate of synthesis is also involved in determining levels of type II hexokinase during diabetes (Frank and Fromm 1986b) and, presumably, starvation-refeeding.

Multiple forms of all of the isozymes, types I–IV, have been detected in various studies. For example, Weinhouse et al. (1972) reported two electrophoretically distinct forms of type I hexokinase in fetal rat liver, and two distinct forms of type IV in liver of rats at 17–21 days postnatally. Ueda et al. (1975) presented ion exchange chromatography results indicating separation of the type I activity into two partially resolved subforms present in extracts of rat liver, while Allen and Walker (1975) reported electrophoretic separation of type IV hexokinase from rat, pig, and baboon liver into *four* distinguishable forms. Rogers et al. (1975) observed that the type I, II, and III isozymes each appeared as two electrophoretically distinguishable components in extracts from several human tissues. As noted earlier, with the exception of the type II_a and II_b forms discussed above, this multiplicity was not seen in the original studies leading to recognition of the various isozymes of hexokinase (Katzen and Schimke 1965; Grossbard and Schimke 1966; Gonzalez et al. 1967). The explanation for this does not seem readily apparent. It might be argued that the resolving power of the separation techniques was enhanced in later studies, permitting detection of species not seen in the earlier work. This seems unlikely since the basic techniques used, most commonly starch gel electrophoresis or DEAE-cellulose ion exchange chromatography, were the same. In view of the probable role for sulfhydryl modification in generating subforms of type II hexokinase (Katzen 1966), and noting the conservation of Cys residues among the mammalian isozymes (Table 2), it is conceivable that sulfhydryl modification may, at least in some cases, account for microheterogeneity seen with the other isozymes, for example, the multiple forms of the type I, II, and III isozymes reported by Roger et al. (1975), who did not include reducing agents in their homogenization or electrophoresis buffers.

Although it had been suggested that there might be two forms of the type I isozyme found in brain (see Wilson 1985), differing in their ability to bind to mitochondria, subsequent studies have not supported this view (Sprengers et al. 1983; Needels and Wilson 1983). "Bindable" and "nonbindable" forms, separable by DEAE-cellulose column chromatography (Felgner and Wilson 1976), ion exchange HPLC (Polakis and Wilson 1982), or isoelectric focusing (Needels and Wilson 1983; Polakis and Wilson 1985), were detected in rat brain extracts. However, the "nonbindable" form was found to result from limited proteolysis at

the N-terminus of the enzyme by endogenous protease activity in the tissue homogenate (Polakis and Wilson 1985). Rapid purification of the enzyme by HPLC (Polakis and Wilson 1982), or maintenance of a mildly alkaline pH to minimize lysosomal protease activity during the purification procedure (Wilson 1989) permitted isolation of highly purified enzyme with retention of the ability to bind to mitochondria.

Magnani et al. (1984a) purified the "cytoplasmic" hexokinase from the soluble fraction of rabbit brain homogenates, comparing it to the purified mitochondrial enzyme. As with the "soluble" and "mitochondrial" hexokinases from rat brain (Needels and Wilson 1983; Sprengers et al. 1983), no differences were found in molecular weight, electrophoretic behavior, or isoelectric point. Magnani et al. did report that, relative to the solubilized mitochondrial enzyme, the cytoplasmic hexokinase had a diminished ability to phosphorylate fructose. They also reported differences in Arrhenius plots for the two activities. In both cases, the Arrhenius plots were biphasic, with breaks (abrupt change in slope) occurring at 28°C and 32°C for the cytoplasmic and mitochondrial enzymes, respectively; slightly higher activation energies were reported for the cytoplasmic hexokinase, 8 kcal/mol and 14 kcal/mol (above and below transition temperature, respectively), compared with 5.9 kcal/mol and 11 kcal/mol for the mitochondrial enzyme. In contrast to these results with the rabbit brain enzyme (Magnani et al. 1984a), the Arrhenius plot obtained with the purified mitochondrial hexokinase from rat brain is monophasic with an activation energy of 11 kcal/mol (Wilson 1971). Magnani et al. (1984a) did not determine if the purified "cytoplasmic" hexokinase could bind to mitochondria. Whether the reported differences are indeed indicative of distinct forms of hexokinase in rabbit brain or the result of modifications during the purification process remains uncertain.

Two subtypes of type I hexokinase have been purified from human placenta (Magnani et al. 1988a), one with an apparent molecular weight of about 112 kDa and a second with a molecular weight of about 103 kDa. The higher molecular weight form was subsequently (Magnani et al. 1991) shown to be missing the first 10 amino acids from the N-terminus while the 103-kDa form had lost 102 residues from the N-terminus. Since the N-terminal sequence is critical for binding to mitochondria (Polakis and Wilson 1985), it is apparent why both of these forms were incapable of binding to mitochondria (Magnani et al. 1988a). Mitochondrially bound type I hexokinase, presumably with an intact N-terminus, was also present in placenta (Magnani et al. 1988a), and in vitro translation of placental mRNA gave a single product with

a molecular weight of about 112 kDa (Magnani et al. 1991). It is thus likely that the purified "soluble" forms represent the result of post-translational proteolytic modification of an intact type I isozyme. Whether these occur in vivo or result from artifactual modification during processing of the tissue (as may occur with brain – see above) is not really clear. Magnani et al. (1991) attempted to address this by showing that both "soluble" forms could be isolated by immunoaffinity chromatography of placental extracts prepared with inclusion of various protease inhibitors in the homogenization buffer. However, given the variety of protease activities likely to be present, there is no assurance that the inhibitors added were effective at inhibiting the protease(s) that might be responsible for the modification; our own experience (unpublished observations) has been that the inhibitors employed by Magnani et al. (1991) were not adequate to prevent modification of the type I isozyme by protease activities present in brain homogenates. Although the mildly alkaline pH of the homogenization buffer, pH 7.55, might have moderated the effect of lysosomal proteases, it is noted that the buffer concentration used by Magnani et al. (1991) was rather low (5 m*M* phosphate), and whether this was adequate to maintain a mildly alkaline pH is uncertain. Nonetheless, Magnani et al. (1991) indicated that the heterogeneity was reproducibly observed in five different placental samples, and thus the possibility that the multiple forms are indeed in vivo realities remains open.

Of all the reports describing multiple forms of type I hexokinase, perhaps the results with red blood cells and their precursors, the reticulocytes, most convincingly indicate the in vivo reality of this microheterogeneity. Two or more distinct forms, separable by ion exchange chromatography or electrophoresis, have been reported in reticulocytes and erythrocytes from a number of species, including human, rabbit, cow, dog, and horse (Rijksen et al. 1977, 1981; Magnani et al. 1982, 1984b, 1988b; Fornaini et al. 1982; Stocchi et al. 1982, 1988), but curiously, not rat (Magnani et al. 1982; Serafini et al. 1986). The relative amounts of the different forms vary during the course of maturation from reticulocytes to erythrocytes and during aging of the latter (Rijksen et al. 1977, 1981; Fornaini et al. 1982, 1985; Magnani et al. 1984b; Stocchi et al. 1982, 1988), presumably the result of posttranslational modifications. Three major forms of the type I isozyme have been purified from human erythrocytes (Magnani et al. 1988b; Stocchi et al. 1982, 1988) and, while differing in isoelectric point, were found to be indistinguishable in molecular weight determined by sucrose density gradient centrifugation or sodium dodecyl sulfate gel electrophoresis.

While the latter result excludes gross modification by proteolysis as a mechanism for generation of the multiple forms, it is apparent that limited proteolysis at either the N- or C-terminus might be involved. As discussed above, removal of only a few amino acids from the N-terminus of the type I isozyme from rat brain has marked effect on the isoelectric point (Polakis and Wilson 1985).

Modification at the N-terminus would also affect ability to bind to mitochondria and it is therefore noteworthy that one (designated type Ib) of two forms of type I hexokinase from rabbit reticulocytes has been found solely in the soluble fraction (Magnani et al. 1984b). Binding to mitochondria has been suggested to protect the enzyme against degradative processes, with preferential loss of the soluble form(s) accounting for the decrease in hexokinase activity that occurs during reticulocyte maturation in rabbits (Magnani et al. 1984b; Stocchi et al. 1988). This does not appear to be of general significance, however, since, unlike the situation in rabbit reticulocytes, none of the hexokinase activity in human reticulocytes has been reported to be mitochondrially bound (Jansen et al. 1985).

Although degradation of hexokinase via an ATP- and ubiquitin-dependent mechanism has been suggested to account for the loss of hexokinase during reticulocyte maturation (Magnani et al. 1986), Thorburn and Beutler (1989) disagree. The latter authors found that degradation by the ubiquitin-dependent pathway appeared to result only after storage of red cell lysates or addition of a free radical generating system to fresh lysates. They therefore concluded that, although oxidatively damaged hexokinase might be susceptible to degradation by the ubiquitin-dependent pathway, this was not likely to be the mechanism by which degradation normally occurred during reticulocyte maturation.

The possible physiological consequences that might result from changes in forms of hexokinase present at various maturational stages is unclear. Most investigators (Rijksen et al. 1977, 1981; Stocchi et al. 1982; Fornaini et al. 1982, 1985) have reported that the various forms are rather similar in their kinetic and regulatory properties, although Rijksen et al. (1981) did note that P_i was much less effective at reversing inhibition of one of the forms found in human erythrocytes by glucose 1,6-diphosphate. This is particularly interesting in light of comments above indicating that loss of sensitivity to P_i was associated with "aging" of the enzyme, which certainly occurs during maturation of erythrocytes.

7.5 The Mammalian Type IV Isozyme

Of all the hexokinases, the mammalian type IV isozyme is currently perhaps the most actively studied. This would be understandable if only because of the importance of this isozyme in glucose metabolism and homeostasis, but recognition that mutations in the type IV hexokinase gene may be associated with certain types of diabetes has brought even greater attention. Excellent reviews by leading investigators in the field are also being published on a regular basis, and the interested reader is referred to the articles by Iynedjian (1993), Printz et al. (1993b), or Randle (1993) for authoritative discussion. Other recent reviews, dealing with particular aspects of type IV hexokinase function, are noted below.

7.5.1 Hepatic Glucokinase

When compared with the other mammalian isozymes of hexokinase, perhaps the most distinctive feature of the type IV isozyme, aside from its 50-kDa molecular weight and lack of sensitivity to inhibition by glucose 6-phosphate, is the cooperativity in binding of glucose as substrate, with an affinity in the several millimolar range (Storer and Cornish-Bowden 1977). Cahill and his colleagues (1958) had shown that hepatic glucose metabolism was responsive to fluctuations of glucose concentrations within the physiological (millimolar) range. Since hepatocytes were found to be freely permeable to glucose, this pointed to the possibility that metabolism is dependent on phosphorylation of glucose by a "glucokinase" having an affinity in the millimolar range, unlike the then-known hexokinases found in, for example, brain (Sols and Crane 1954). The presence of such an activity in liver, and its importance as a regulator of hepatic glucose metabolism, was subsequently demonstrated by DiPietro et al. (1962). The original concept was that increased glucose phosphorylation by liver glucokinase, in response to increased plasma glucose levels (e.g., postprandial), directed the metabolism of the carbohydrate into storage reserves as glycogen or fat. Subsequent work indicated, however, that even in the presence of a relative abundance of glucose, direct incorporation of the carbohydrate into hepatic glycogen or lipid stores was limited, a surprising finding termed the "glucose paradox" (Katz et al. 1986). It was suggested (Kuwajima et al. 1986) that hepatic levels of glucokinase were inadequate to phosphorylate glucose at a rate adequate to provide substrate for synthesis of storage forms, but this was contested and attributed to assay of glucokinase

activities under less than optimal conditions (Davidson and Arion 1987). Of course, the relationship between activities determined in vitro and actual activity of an enzyme in situ is always an open question. Whatever the case, it now seems established (Kurland and Pilkis 1989) that appreciable synthesis of hepatic glycogen occurs through an "indirect" route, in which glucose is first metabolized to 3-carbon compounds such as lactate; the extent to which the latter occurs within the liver or in extrahepatic tissues seems to be unclear (Katz et al. 1986; Kurland and Pilkis 1989). The 3-carbon metabolites are then reconverted to glucose via the gluconeogenic pathway, and ultimately to glycogen. Thus, hepatic glucose metabolism is likely governed by factors more complex than simply availability of substrate glucose (Kurland and Pilkis 1989), although the latter is surely an important influence.

Kinetically, hepatic glucokinase is well-suited for responding to changes in plasma glucose concentrations. This is likely to be a major factor regulating in vivo glucokinase activity but other factors may also be operational. For example, Tippett and Neet (1982a,b) noted that the enzyme was inhibited by monomeric (i.e., not the micellar form) of long chain acyl coenzyme A derivatives; although these authors speculated about the possible physiological significance, others (Vandercammen and Van Schaftingen 1991; Printz et al. 1993b) have offered the view that in vivo concentrations of free acyl coenzyme A derivatives are unlikely to be in a range in which they would be a significant regulatory factor.

Van Schaftingen (1989) observed that fructose 1-phosphate stimulated glucokinase activity in extracts of soluble proteins from rat liver. This effect was shown to be dependent on a protein that was separated from glucokinase by anion exchange chromatography. The protein was subsequently purified (Vandercammen and Van Schaftingen 1990) and shown to exert its inhibitory effect by complexing with glucokinase. Fructose 1-phosphate promoted dissociation of the complex and hence activation of the enzyme, whereas complexation, and thus inhibition, was favored in the presence of fructose 6-phosphate. The effects of the inhibitory protein were competitive with palmitoyl coenzyme A, which was taken to indicate that these two effectors compete for a common site on glucokinase (Vandercammen and Van Schaftingen 1991); the latter authors argued that inhibition by what they viewed as unphysiologically high concentrations of acyl coenzyme A derivatives resulted simply from their ability to bind at the site for the regulatory protein, the latter being the relevant effector in vivo.

Studies with a series of fructose 1- or 6-phosphate analogs (Detheux et al. 1991) provided further insight into the structural requirements for

inducing conformations of the regulatory protein that did or did not complex with glucokinase. Kinetically, the regulatory protein behaved as a competitive inhibitor versus glucose, and it was suggested that binding (promoted by fructose 6-phosphate) of the regulatory protein at an allosteric site induced conformational changes that precluded binding of glucose at the catalytic site (Vandercammen and Van Schaftingen 1991). It might be noted that this is analogous to the mechanism proposed (White and Wilson 1990) to explain inhibition, competitive versus ATP, of the type I (and possibly type II and type III) hexokinase by glucose 6-phosphate, i.e., binding of glucose 6-phosphate to the N-terminal half induces a conformational change that precludes binding of substrate ATP to the C-terminal half (Fig. 4). In this view, the regulatory protein becomes analogous to the N-terminal half of the 100-kDa type I isozyme. Schwab and Wilson (1991) speculated that the regulatory protein of Van Schaftingen might, in fact, represent the equivalent of the N-terminal half of a 100-kDa hexokinase, the gene for which had resplit (see discussion in Sect. 3.1.3) to give rise to genes encoding type IV hexokinase plus the regulatory protein. However, the cDNA for the regulatory protein has now been cloned (Detheux et al. 1993) and the deduced amino acid sequence shows no similarity to the hexokinases (Fig. 2), in contrast to what would have been predicted from the scenario entertained by Schwab and Wilson (1991).

The regulatory protein has been shown to be present in liver from a number of species, but absent from several other tissues which do not express glucokinase activity and from liver in species which do not express hepatic glucokinase (Vandercammen and Van Schaftingen 1993). In rats, changes in levels of the regulatory protein more or less paralleled changes in glucokinase activity during development, during starvation and refeeding, and in response to streptozotocin-induced diabetes and subsequent insulin treatment (Vandercammen and Van Schaftingen 1993). As is discussed below, a variant of hepatic glucokinase is expressed in pancreatic islets. Malaisse et al. (1990a) reported that activity of pancreatic glucokinase was influenced by a regulatory protein analogous to that found in liver. However, pancreas was not included in the tissues studied by Vandercammen and Van Schaftingen (1993) and hence direct demonstration of an immunologically crossreacting (with antibodies against the liver regulatory protein) regulatory protein in pancreas has not yet been provided. The apparent close correlation between expression of glucokinase and its regulatory protein led Vandercammen and Van Schaftingen (1993) to suggest that these should be viewed as a "functional unit."

As mentioned earlier, the type IV isozyme does not possess the hydrophobic N-terminal sequence that is required for binding to mitochondria (Polakis and Wilson 1985; Gelb et al. 1992), and the prevailing view has been that glucokinase is located in the cytoplasm (Iynedjian 1993). Sometimes implicit in a "cytoplasmic" location is the assumption that the enzyme, in this case, glucokinase, is relatively free from interactions with other cellular components that would result in specific organizational patterns of functional significance; this assumption is, however, increasingly and justifiably questioned (Ovádi 1991). In this context, the recent work of Agius and Peak (1994) is of particular interest. Based on rate of release of glucokinase and other enzymes from cultured rat hepatocytes permeabilized with digitonin, these authors concluded that, with hepatocytes cultured in low (5 m*M*) glucose, glucokinase was associated with some intracellular component that impeded its release from permeabilized cells. Preincubation of the cells with elevated (10–30 m*M*) glucose or low (50 μM–1 m*M*) fructose resulted in increased rate of release. This was interpreted as indicating ambiquitous behavior of glucokinase, with "translocation" from a bound form to a free form in response to fructose or elevated glucose. Agius and Peak (1994) suggested that altered binding of glucokinase might be involved in directing glucose 6-phosphate toward either glycolytic metabolism or glycogen synthesis. Confirmation of this interpretation by alternative methods, and assessment of its possible functional significance, is of obvious interest and importance.

The genes for the rat (Magnuson et al. 1989) and human (Stoffel et al. 1992) type IV hexokinases have been isolated and characterized. The human gene has been localized to chromosome 7, band p13 (Matsutani et al. 1992; Nishi et al. 1992). The overall intron/exon structure of the rat and human genes is similar, as is nicely illustrated in the reviews of Printz et al. (1993b) and by Iynedjian (1993); these may be consulted for extensive discussion of glucokinase gene structure and factors governing its expression, which are only briefly treated here. Coordination of the expression of glucokinase with that of other proteins involved in hepatic glucose transport and metabolism has been succinctly described by Granner and Pilkis (1990).

Expression of glucokinase activity in liver is known to be influenced by a number of developmental, dietary, and hormonal factors. Generally these seem to act at the transcriptional level. Increased hepatic glucokinase mRNA levels, attributable to increased rates of transcription with little or no effect on message stability, are seen in response to insulin (Spence 1983; Sibrowski and Seitz 1984; Iynedjian et al. 1987, 1988,

1989a; Magnuson et al. 1989; Nouspikel and Iynedjian 1992); it is not clear whether insulin acts directly or through induction of some factor(s) affecting transcription of the glucokinase gene (Nouspikel and Iynedjian 1992). Conversely, glucagon represses transcription of the glucokinase gene via a mechanism involving 3′,5′-cyclic AMP (Iynedjian et al. 1989a; Nouspikel and Iynedjian 1992); Sibrowski and Seitz (1984) had previously demonstrated the repressive effect of cAMP on levels of mRNA for hepatic glucokinase. Developmental increases in hepatic glucokinase levels have been associated with the change from the high fat milk diet to a high carbohydrate diet at weaning, and can be advanced or retarded by manipulations that influence carbohydrate intake (Haney et al. 1986; Girard et al. 1992; Bossard et al. 1993), presumably through the effect that carbohydrate intake would have on plasma insulin and glucagon levels (Narkewicz et al. 1990; Girard et al. 1992). The effects of insulin and glucagon are, however, influenced by other hormones. Thus, thyroid hormones have been said to be "permissive" for the stimulation of expression by insulin (Höppner and Seitz 1989), with insulin-induced increases in mRNA for hepatic glucokinase being much less in thyroidectomized animals (Minderop et al. 1987; Höppner and Seitz 1989); triiodothyronine itself can induce increased levels of glucokinase mRNA in hepatocytes (Narkewicz et al. 1990). Glucocorticoids do not themselves induce increased transcription but do enhance the response to insulin, apparently by increasing the stability of glucokinase mRNA (Minderop et al. 1987; Narkewicz et al. 1990). Surprisingly, dexamethasone did not enhance, but rather diminished, the increase in mRNA seen after administration of thyroid hormone to thyroidectomized animals (Narkewicz et al. 1990); this is still not understood but clearly indicates the existence of rather complex hormonal interactions in regulating expression of hepatic type IV hexokinase. The effects that altered nutritional status, e.g., starvation or carbohydrate-free diet (Reyes et al. 1984), have on hepatic glucokinase levels are likely mediated through effects on hormonal levels. However, a more direct effect of nutritional factors may also be operative. Thus, administration of biotin led to increase in both glucokinase activity and levels of its mRNA in liver of starved rats, and these effects were shown to be attributable to increased transcriptional activity (Chauhan and Dakshinamurti 1991).

Elucidation of regulatory elements in the 5′-flanking region of the glucokinase gene that might govern specific expression in liver or the response to hormones such as insulin has proven to be quite challenging. Transfection of hepatocytes with constructs containing up to 7 kb of the 5′-flanking region linked to reporter genes has not provided clear

indication of functional regulatory elements (Noguchi et al. 1989; Iynedjian 1993; Printz et al. 1993b). Differences in hepatic glucokinase activity and mRNA levels in inbred mouse strains have been suggested to be attributable to differences in regulation of expression of the glucokinase gene (Middleton and Walker 1992); definition of specific mechanism(s) by which this might occur obviously will require understanding of the regulatory elements that are involved.

The distribution of glucokinase (and other enzymes) is not homogeneous within the liver (Jungermann 1992). In adult rat liver, histochemical and immunohistochemical (Lawrence et al. 1984; Kirchner et al. 1993) as well as microdissection (Fischer et al. 1982) studies have indicated that glucokinase levels are considerably higher, perhaps by about 3.5-fold (Fischer et al. 1982), in perivenous (pericentral) regions compared to periportal regions. However, the studies of Kirchner et al. (1993) suggest that a quite different pattern may be seen in the developing liver, with higher levels in periportal regions during the first month postnatally. It is also interesting to note that even within the perivenous region there appears to be considerable heterogeneity in levels of glucokinase among the hepatocyte population (Lawrence et al. 1984).

The metabolic implications of this uneven distribution between perivenous and periportal regions seem to be in dispute. Although elevated levels of glucokinase in perivenous hepatocytes might reasonably be interpreted as adapting these cells for avid metabolism of glucose to glycogen or through the glycolytic pathway (Jungermann 1992), others (Chen and Katz 1988) suggest that this is not the primary role of glucokinase in liver. Matschinsky (1990) has proposed that hepatic glucokinase functions as a glucose sensor (analogous to its role in pancreas, discussed below), modulating hepatic metabolism in response to altered plasma glucose levels but without flux through the glucokinase step itself being quantitatively important as a supplier of precursors for glycogen or lipid synthesis.

The distribution of glucokinase is only partially mirrored by the distribution of its mRNA. Thus, oral glucose loading or refeeding of starved rats does preferentially increase perivenous levels of glucokinase mRNA, as determined by in situ hybridization (Moorman et al. 1991a; Eilers et al. 1993). However, as the situation returns to normal, the distribution of the mRNA becomes rather uniform, despite the existence of higher levels of glucokinase itself in perivenous regions. This suggests some role for posttranscriptional mechanisms in determining glucokinase levels. It should be mentioned, however, that both Moorman et al. (1991a) and Eilers et al. (1993) expressed some caution in interpreting

the in situ hybridization patterns, noting that technical limitations might have precluded detection of relatively subtle differences between mRNA levels in perivenous and periportal regions.

Moorman et al. (1991b) have also used in situ hybridization to determine the distribution of mRNA for the CCAAT/enhancer binding protein (C/EBP) in liver of normal or dexamethasone-treated rats and after refeeding of starved rats. Based on a spatial correlation with the distribution of mRNA for glucokinase, it was suggested that C/EBP plays a role in regulating the expression of hepatic glucokinase.

7.5.2 Pancreatic Glucokinase

Secretion of insulin by the pancreatic β-cells is an important factor in maintaining plasma glucose levels within a relatively narrow range. The mechanism by which insulin secretion is coupled to changes in plasma glucose levels has attracted considerable interest for many years. Articles by Matschinsky (1990), Matschinsky et al. (1993), Randle (1993), and Mueckler (1993) provide concise reviews of events leading to the currently favored view that glucokinase plays a central role as "glucose sensor" in pancreatic β-cells.

Transport of glucose into both hepatocytes and pancreatic β-cells (Johnson et al. 1990) is mediated by the glucose transporter commonly referred to as GLUT2. This transporter is characterized by a high transport capacity but relatively low (compared to other glucose transporters in mammalian tissues) affinity for glucose, having a "K_m" of approximately 15 m*M*. The net result is that intracellular glucose levels in both hepatocytes and pancreatic β-cells are thought to rapidly equilibrate with, and hence be responsive to changes in, plasma glucose levels in the physiological range of 4–10 m*M*. As noted above, glucose phosphorylation by hepatic glucokinase is dependent on glucose concentrations in this range. Thus, an early postulation was that a similar enzyme might exist in pancreas, with metabolism of glucose, governed by the rate of phosphorylation by a pancreatic glucokinase, being linked to insulin secretion (reviewed by Matschinsky 1990, Matschinsky et al. 1993, and Randle 1993). The presence of glucokinase in pancreatic islets and β-cell tumors was unequivocally demonstrated by Meglasson et al. (1983a,b), and subsequent studies (e.g., Shimizu et al. 1988; Liang et al. 1992) have provided strong support for the concept that insulin secretion is linked to metabolism of glucose, with glucokinase acting as glucose sensor.

That glucose phosphorylation is indeed the determinative step in insulin secretion is supported by the recent work of Epstein et al. (1992).

Transgenic mice, expressing yeast hexokinase (recall that yeast hexokinase, as the mammalian type IV isozyme, is not sensitive to inhibition by glucose 6-phosphate) under the control of an insulin promoter, showed elevated hexokinase activity in pancreatic islets but not in other tissues examined. The transgenic animals also showed elevated plasma insulin levels and decreased plasma glucose levels.

Still further support for the role of glucose phosphorylation in regulation of insulin synthesis comes from the study of German (1993). Rat pancreatic β-cells were transfected with a plasmid encoding type I hexokinase, and cotransfected with a chloramphenicol acetyltransferase (CAT) gene regulated by an insulin promoter which served as a reporter gene. CAT expression in the cotransfected cells was maximal at 1 m*M* glucose in the culture medium while control cells, which did not receive the plasmid encoding type I hexokinase, required much higher concentrations of glucose to elicit expression of CAT. These results are consistent with phosphorylation of glucose being the key regulatory element, transfection with the low K_m type I hexokinase eliciting a response at glucose concentrations that were relatively ineffective with control cells containing predominantly the high K_m glucokinase. The cotransfected cells also showed expression of CAT activity in response to fructose while control cells did not, consistent with the substrate specificity of the type I and type IV isozymes, the latter being much less effective at phosphorylating fructose. Cells grown on pyruvate in place of glucose or fructose showed markedly decreased expression of CAT, and pyruvate itself was rather ineffective at evoking CAT expression in glucose-grown cells. However, after cotransfection with a plasmid encoding phosphoenolpyruvate carboxykinase (which should permit production of phosphoenolpyruvate from the tricarboxylic acid cycle intermediate, oxaloacetate), pyruvate did evoke an almost fivefold increase in CAT expression. German (1993) interpreted these results as indicating that some intermediate between fructose 1,6-bisphosphate and phosphoenolpyruvate in the glycolytic pathway was essential for insulin expression. German (1993) also cotransfected the cells with a plasmid encoding the GLUT1 glucose transporter, either with or without type I hexokinase, but found no effect on CAT expression, suggesting that glucose phosphorylation, not glucose transport, was the critical step.

Thus, there is considerable evidence to support the view that pancreatic glucokinase functions as a sensor governing insulin synthesis and secretion in response to altered plasma glucose levels. Less clear is the mechanism by which the latter processes are linked to glucose metabolism. One notion, the "fuel concept" (Lenzen 1990; Malaisse et al.

1990b), attributes glucose-stimulated insulin release to increased levels of ATP resulting from increased rate glucose metabolism. This is thought to lead to closure of ATP-sensitive K^+ channels in the plasma membrane, depolarization of the plasma membrane, opening of voltage sensitive Ca^{2+} channels with resulting influx of Ca^{2+}, and Ca^{2+}-induced exocytosis of insulin. Cited as support for this is the observation that alternative nonphysiological (i.e., not normally present at effective concentrations in plasma) substrates for energy metabolism, such as mannose or glyceraldehyde, also stimulate insulin release from islets; thus, insulin secretion is not dependent on metabolism of glucose per se. However, Ghosh et al. (1991) reported that ATP levels were unchanged when insulin release was stimulated by perfusion of the pancreas with elevated glucose, although there did seem to be a modest decrease in "free" (cytoplasmic) ADP. Matschinsky et al. (1993) suggest that it is the latter that is of primary importance, with decreases in ADP leading to closure of the ADP-activated K^+ channels and subsequent insulin secretion by a chain of events otherwise identical to those outlined just above. Whether it is ATP or ADP – or some other metabolite – that is of central importance for insulin secretion remains to be established.

As mentioned above, German (1993) found CAT expression under control of an insulin promoter to be enhanced by growth on glucose or fructose but much less so with pyruvate as growth substrate. It seems reasonable to expect that ATP and ADP levels would be similar with any of these substrates, since they all support metabolism through the tricarboxylic acid cycle and mitochondrial oxidative phosphorylation, the predominant source of ATP production. This would seem to argue against either ATP or ADP being directly responsible for regulation of insulin synthesis. Thus, if either nucleotide is involved in regulating secretion, some additional mechanism for coupling synthesis with secretion seems likely.

Pancreatic islets (Meglasson et al. 1983a) and β-cell tumors (Meglasson et al. 1983b) also contain "low K_m" hexokinase activity, probably the type I isozyme (Matschinsky et al. 1993). Although, in principle, this could contribute to glucose phosphorylation and hence insulin secretion, it appears to be the case that the low K_m activity contributes relatively little, presumably due to extensive inhibition by intracellular levels of glucose 6-phosphate (Shimizu et al. 1988; Liang et al. 1992). The results of German (1993) are consistent with this view.

As noted above, Lawrence et al. (1984) pointed out the heterogeneity in the perivenous hepatocyte population, with some cells staining intensely for glucokinase and others barely staining at all. It is inter-

esting that an analogous situation has been reported for the pancreatic **b**-cell population (Jetton and Magnuson 1992), with some cells staining much more intensely than others. In contrast, staining for insulin and for the GLUT2 glucose transporter was rather uniform among **b**-cells. Heterogeneity in glucokinase content was suggested to underly functional heterogeneity among the β-cell population, i.e., differences in the insulin secreting response evoked by altered extracellular glucose levels. Heimberg et al. (1993) have provided results in accord with this suggestion, showing enhanced synthesis of proinsulin in response to elevated glucose in **b**-cells containing higher glucokinase activities; the latter cells also had higher levels of glucokinase mRNA. Heimberg et al. (1993) found no discernible difference in levels of the GLUT2 glucose transporter or its mRNA in cells that differed in their response to increased glucose as a stimulus for proinsulin synthesis. Thus, in accord with others (Jetton and Magnuson 1992; German 1993), Heimberg et al. (1993) conclude that heterogeneity among **b**-cells in their response to glucose is attributable to differences in capacity for glucose phosphorylation, not transport.

The studies of MacDonald et al. (1991) complicate what might otherwise be considered a rather nice correlation between glucose phosphorylation/metabolism and insulin secretion. They report that culture of rat pancreatic islets for 24 h in low (1 m*M*) glucose resulted in loss of the glucose-induced insulin release response *without* decrease in glucose phosphorylating activity. These observations may be contrasted with those of Liang et al. (1992) who reported marked decrease in the insulin response *and* glucokinase activity after culture of islets for 24 h in low (3 m*M*) glucose. Liang et al. (1992) also reported that glucose utilization by cultured islets was dependent on glucose concentration in the medium while MacDonald et al. (1991) found that islets cultured at low- and high-glucose conditions did not differ in their rate of glucose utilization; in both studies, glucose metabolism was measured by loss of tritium from [5-^{3}H]-glucose. MacDonald et al. (1991) concluded that glucose concentration in the culture medium (at least for the one day culture period) had no effect on glycolytic metabolism of glucose at least as far as phosphoenolpyruvate, and attributed the loss of the insulin response to effects of the culture conditions on enzymes of mitochondrial metabolism. It seems surprising that such fundamental differences in experimental results have not merited discussion in recent reviews on this subject (e.g., Randle 1993; Matschinsky et al. 1993). Clearly there is much more to learn about the linkage between metabolism and insulin secretion from **b**-cells.

Studies with the hepatic and pancreatic glucokinases had provided no basis for viewing them as distinct species. On the contrary, they appeared to be similar in a number of their properties (Meglasson et al. 1983a,b; Vischer et al. 1987). It was therefore surprising to find that the mRNAs for glucokinase in rat liver and pancreas (or pancreatic tumor cells) were distinct, the pancreatic mRNA being about 400 bp longer than the hepatic form (Magnuson and Shelton 1989; Iyndejian et al. 1989b). This was shown to be due to use of alternate promoters for transcription of a single glucokinase gene in a tissue specific manner; the promoter responsible for transcription of the pancreatic form was located at least 12 kb upstream from the hepatic promoter, and hence the β-cell promoter is also referred to as the "upstream promoter." In contrast to the situation with expression from the hepatic (downstream) promoter (Noguchi et al. 1989; Iynedjian 1993; Printz et al. 1993b), analysis of regulatory elements governing expression from the upstream promoter has progressed to some extent, with Magnuson and Jetton (1993) providing a concise summary of current status.

The only difference in amino acid sequence is at the N-terminus of the enzyme, with 11 of the first 15 residues differing in the rat pancreatic and hepatic forms (Magnuson and Shelton 1989):

Pancreatic glucokinase: **MKDDRARMEATKKEK**
Hepatic glucokinase: **MAMDTTRCGAQLLTL**

An analogous relationship exists between pancreatic and hepatic forms of human glucokinase (Koranyi et al. 1992). The possible functional significance of these sequence differences remains unclear. They do not appear to affect kinetic or other properties of the enzyme that have been examined. It should be noted that the N-terminal sequence of the pancreatic form is, as that of the hepatic glucokinase, *not* hydrophobic in character. Thus, if reports of binding of pancreatic glucokinase to mitochondria (Sener et al. 1986; Malaisse-Lagae and Malaisse 1988) are confirmed, it is evident that it cannot be in the same manner as binding of the type I isozyme, for which a hydrophobic N-terminal segment is clearly essential (Polakis and Wilson 1985; Gelb et al. 1992).

The use of different promoters is consistent with the differences in regulation of glucokinase expression in liver and pancreas (Bedoya et al. 1986; Iynedjian et al. 1989b). As discussed above, expression of hepatic glucokinase is regulated at the transcriptional level by a number of hormones, with insulin and glucagon perhaps being of primary importance as mediators of the response to altered nutritional or dietary status. It was originally reported that refeeding after starvation increased mRNA for hepatic glucokinase but had no effect on pancreatic glu-

cokinase mRNA levels (Iynedjian et al. 1989b); however, Tiedge and Lenzen (1991) found that mRNA levels in *both* tissues respond in a similar manner to fasting-refeeding. The latter workers also reported that changes in levels of mRNA for the GLUT2 glucose transporter in response to altered nutritional status were correlated with those for glucokinase in both liver and pancreas; the latter observation is consistent with the suggestion of Bell et al. (1993a) that specific isozymes of hexokinase might be associated in a functional manner with specific forms of the mammalian glucose transporter. Tiedge and Lenzen (1991) also observed a larger (about 4.4 kb) mRNA in β-cells, in addition to the approximately 2.8 kb mRNA seen by Iynedjian et al. (1989b) and Magnuson and Shelton (1989); the larger species had previously been detected by Iynedjian et al. (1989b) in β-cell tumor cells but not in normal pancreas. Trace amounts of a larger mRNA species were also seen in liver after refeeding of fasted rats but not in normal rat liver (Tiedge and Lenzen 1991). The significance of the larger mRNA species and their relationship to the smaller species predominant in liver and pancreas remains unclear.

Factors involved in the transcriptional regulation of pancreatic glucokinase are much less well defined than in the case of the hepatic enzyme. Glucose itself may be a primary agent in regulation of pancreatic glucokinase activity (Bedoya et al. 1986; Liang et al. 1990, 1992; Magnuson 1990; Purrello et al. 1993), but glucose-induced increases in glucokinase activity were not found to be associated with increases in levels of the corresponding mRNA (Liang et al. 1992). This suggested that glucose acted by a posttranscriptional mechanism. However, since refeeding would be associated with increased plasma glucose levels, the increased mRNA levels reported by Tiedge and Lenzen (1991) to result from refeeding suggest that an effect at the transcriptional level might also occur. Marie et al. (1993) recently reported that glucokinase mRNA levels were not increased in glucose stimulated INS-1 cells, a β-cell tumor line. However, to this observer, glucose stimulation *did* appear to produce a readily detectable increase in the intensity of signal on northern blots probed for glucokinase mRNA (see Fig. 2 in Marie et al. 1993); the effect was transient, with below normal levels seen after 24 h of culture in the presence of elevated glucose concentration.

Transcription from the upstream promoter (β-cell) also occurs in anterior pituitary cells or in derived cell lines designated AtT20 or $AtT20_{ins}$, the latter representing an anterior pituitary cell line, AtT20, that had been stably transfected with the insulin gene (Hughes et al. 1991). Low levels of an approximately 50-kDa protein were detected by

western blotting of extracts from normal anterior pituitary tissue, but no glucokinase activity was detectable. Intensity of staining of an approximately 50-kDa protein was more prominent on Western blots of $AtT20_{ins}$ cell extracts, and significant enzymatic activity was detected – though well below activities seen in liver (Bedoya et al. 1986; Iynedjian et al. 1989b) or pancreatic islets (Bedoya et al. 1986). In addition to the major forms of glucokinase mRNA, a number of variant mRNAs resulting from alternative splicing have been found in pancreatic islets (Hughes et al. 1991) and related tumor cells (Magnuson and Shelton 1989), liver (Hayzer and Iynedjian 1990), and anterior pituitary cells or derived tumor cell lines (Hughes et al. 1991). The net result in each of these variants is deletion of a significant number of residues from within the coding sequence, with detrimental effects on catalytic function expected, or a frameshift resulting in premature truncation of the coding sequence (Magnuson 1990; Printz et al. 1993b; Iynedjian 1993). It is therefore not surprising that expression of these variant forms in *E. coli* results in the absence of detectable glucokinase activity (Liang et al. 1991; Quaade et al. 1991). Since only a variant glucokinase mRNA coding for a truncated protein was detected in anterior pituitary tissue, this would explain the absence of detectable glucokinase activity (Hughes et al. 1991). Hughes et al. (1991) did point out, however, that detection of an approximately 50-kDa protein on western blots, presumably glucokinase, implies the presence of significant mRNA coding for the full length protein in anterior pituitary cells. They suggested that the levels were too low to be detected, or that the full length mRNA was processed to the variant form during removal or processing of the anterior pituitary tissue; extremely low levels seems more likely since further postmortem processing of a "complete" mRNA in this manner seems unprecedented. In any case, the physiological significance, if any, to be attached to expression from the upstream promoter in anterior pituitary cells, or to the occurrence of variant forms of glucokinase mRNA in liver, pancreas, and anterior pituitary, remains unclear.

It is of interest that, unlike liver or pancreas, expression of glucokinase in anterior pituitary cells or in the derived tumor cell line $AtT20_{ins}$ was not accompanied by expression of GLUT2 (Hughes et al. 1991). As noted above, GLUT2 is thought to function in conjunction with glucokinase to modulate pancreatic insulin secretion in response to altered plasma glucose levels. It is therefore not unexpected that insulin secretion from $AtT20_{ins}$ cells is not responsive to altered extracellular glucose levels (Hughes et al. 1992). Hughes et al. (1992) showed that glucose stimulated insulin secretion could be evoked in $AtT20_{ins}$ cells that had

been stably transfected with the GLUT2 cDNA. This was viewed as an initial step in development of an "artificial β-cell" that might find therapeutic application in treatment of type I (insulin-dependent) diabetes.

Based on his proposal that glucokinase functioned as a glucose sensor in pancreas, mediating the secretion of insulin in response to altered plasma glucose levels, Matschinsky (1990) predicted that "certain forms of diabetes mellitus might be due to glucokinase deficiencies in pancreatic β-cells, hepatocytes, or both." This prediction was soon fulfilled by the work of Froguel et al. (1992) who reported close linkage of the glucokinase locus on chromosome 7p with a form of non-insulin-dependent diabetes mellitus (NIDDM), also referred to as type 2 diabetes, in a group of French subjects. Patients in this group were afflicted with a form of type 2 diabetes that is designated "maturity-onset diabetes of the young" (MODY) and characterized by an autosomal dominant inheritance pattern. Subsequent work identified several mutations in the glucokinase gene that resulted in either truncated forms of the enzyme or amino acid substitutions at various points within the sequence (Vionnet et al. 1992; Stoffel et al. 1992; Froguel et al. 1993; Gidh-Jain et al. 1993; Takeda et al. 1993). Mutations associated with MODY have been shown to affect the catalytic function of glucokinase, decreasing V_{max} or the affinity for glucose (Gidh-Jain et al. 1993; Takeda et al. 1993). Patients with identified mutations in the glucokinase locus exhibit abnormal insulin secretion in response to glucose load (Velho et al. 1992). Following the logic of Matschinsky (1990), this may be attributed to decreased glucose phosphorylation, and hence insulin secretion, at a given plasma glucose concentration, i.e., a higher "set point" for glucose-stimulated insulin secretion. MODY has also been associated with mutations that result in abnormal splicing within the glucokinase transcript (Sun et al. 1993). Several recent reviews provide more comprehensive discussion of the relationship between mutations in the glucokinase gene and type 2 diabetes (Permutt et al. 1992; Bell et al. 1993b; Hattersley and Turner 1993; O'Rahilly 1993; Matschinsky et al. 1993).

Since all of the mutations that have been associated with MODY lie within the coding region common to hepatic and pancreatic forms of glucokinase (see above), it can be anticipated that these mutations would also affect glucokinase activity in liver. Markedly depressed hepatic glucose uptake after glucose loading was reported in a MODY patient, suggesting that diminished hepatic glucose uptake may contribute to the hyperglycemia associated with this disorder (Sakura et al. 1993). This apparently is not a major problem, however, since overt signs

of hepatic malfunction are not associated with MODY. Presumably this results from the autosomal dominant inheritance pattern, with coexpression of normal and mutant forms of glucokinase in both liver and pancreas (Bell et al. 1993b). While diminished pancreatic glucokinase activity may be critical for insulin secretion that is closely coupled to the rate of glucose phosphorylation, the effect in liver apparently is less significant. As discussed above, flux through the glucokinase step may not be a major entry point into hepatic carbohydrate and lipid metabolism, and diminished glucokinase activity may therefore have relatively little effect on these major metabolic functions.

Glucokinase mutations are not associated only with MODY. For example, they have been suggested to account for a subset of patients experiencing gestational diabetes, a form of NIDDM in which appearance of symptoms is correlated with early pregnancy (Stoffel et al. 1993). However, NIDDM is a heterogeneous disorder, and it is clear that glucokinase mutations can by no means be cited as the basic problem in the various subforms of the disease (Zouali et al. 1993). This appears to be highly dependent on the racial or geographical subpopulation examined (Chiu et al. 1992, 1993; Cook et al. 1992; Sakura et al. 1992; Froguel et al. 1993; McCarthy et al. 1993; Noda et al. 1993; Shimada et al. 1993).

8 Closing Comments

It is commonplace to muse about the "exponential" increases in scientific information, but generally speaking, it is not evident that such musings lead to significant advances in insight. Therefore, while not pretending that the following comments add substantively to what has gone before, it may nonetheless be appropriate to provide some more subjective remarks about developments in the study of hexokinases since this field was last extensively reviewed (Wilson 1985).

At that time no sequences were known for any of the mammalian enzymes and the postulated evolutionary relationship (Colowick 1973) between the mammalian enzymes and yeast-type hexokinases remained speculation. Subsequent cloning of cDNAs for the 100-kDa mammalian enzymes provided deduced amino acid sequences that confirmed the internal repetition predicted by Colowick's hypothesis. However, the concept that the regulatory site binding glucose 6-phosphate evolved from a duplicated catalytic site was dealt a mortal blow with the finding that

sensitivity to inhibition by glucose 6-phosphate was intrinsic to the "catalytic" C-terminal half of the enzyme (White and Wilson 1989). Definition of the precise location of the functional inhibitory site, and the mechanism by which inhibition occurs, remain as important prerequisites for understanding the molecular basis for regulation of the 100-kDa mammalian enzymes. It is evident that this would be greatly facilitated by determination of an actual structure, and it is thus encouraging that the rat type I hexokinase has now been crystallized in our laboratory, although the crystals are not yet of a quality acceptable for X-ray studies. Nonetheless, it is a start, and perhaps by the time of the next review, speculation about the structure of the mammalian enzyme and reliance on models based on a structure of yeast hexokinase, which is itself still in need of further refinement, will be unnecessary.

It should also be apparent that we have a lot to learn about the "biology" of the 100-kDa mammalian isozymes. It is now clear that acquisition of sensitivity to inhibition by glucose 6-phosphate – acknowledged as an important regulatory influence – does *not* depend on gene duplication/fusion. Thus, if *S. mansoni* (A.G.M. Tielens, personal communication), *S. mobilis* (Viikari 1988), silkworm (Yanagawa 1978), starfish and other marine organisms (Mochizuki and Hori 1977; Mochizuki 1981; Stetten and Goldsmith 1981; Rees et al. 1989) can make do with a *50-kDa* glucose 6-phosphate sensitive hexokinase, what is the evolutionary advantage in having the 100-kDa isozymes characteristic of mammalian tissues? The answer to this question will probably be complex. Development of more sophisticated regulatory responses, including the response to P_i, seems likely to be involved (Wilson 1985; White and Wilson 1989), as does development of capability for specific interaction with subcellular structures such as mitochondria or nuclei. While duplication of genes to code for different isozymes of hexokinase, and acquisition of promoter regions allowing expression of isozymes in a tissue- and hormone-specific manner seems a reasonable expectation, it does not seem evident why this could not have been accomplished with 50-kDa hexokinases. Perhaps the present article will serve as a useful summary of current status, and provoke some of its readers to future work that will enhance our understanding of this intriguing family of enzymes.

Acknowledgements. The continuing financial support provided by NIH Grant NS 09910 has made the work in our laboratory possible and is gratefully acknowledged. That effort has involved a number of undergraduate and graduate students as well as postdoctoral associates, too numerous to list here. The work of many of them is explicitly

mentioned in the present review, but a have contributed in substantial ways to our understanding of hexokinase structure and function. To all of these colleagues, I express my sincere appreciation.

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Editor-in-charge: Professor D. PETTE

Molecular Chaperones and Intracellular Protein Translocation

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Contents

1 Introduction 200

2 Major Classes of Proteins Involved in Protein-Folding Reactions 202
2.1 The DnaK, DnaJ, GrpE System 202
2.2 Chaperonins and TCP1 205
2.3 Hsp90 208
2.4 Hsp104 209
2.5 Small Hsps 209
2.6 Peptidyl-Prolyl Isomerases 209
2.7 Protein Disulfide Isomerases 211

3 Translocation Competence and Folding of Precursor Proteins in the Cytosol 212
3.1 Hsp70s and the Preservation of Transport Competence of Preproteins 212
3.2 DnaJ Homologs 215
3.3 The ATP Requirement of Translocation-Competent Preproteins in the Cytosol 217
3.4 Chaperone Activities in Targeting 219
3.5 Import of Proteins into the Nucleus 222

4 Translocation of Preproteins Across Organelle Membranes 225
4.1 Mitochondria 225
4.1.1 The Conformation of Preproteins During Translocation 225
4.1.2 Hsp70 and the Mechanism of Translocation 227
4.1.3 ATP and the Energetics of Protein Unfolding and Translocation 233
4.2 Chloroplasts 234
4.3 Endoplasmic Reticulum 235

5 Folding and Sorting of Proteins Inside Organelles 238
5.1 Protein Folding and Assembly in the ER 239
5.2 The Sequential Action of Hsp70 and Hsp60 in Mitochondria 242
5.3 Protein Folding in Chloroplasts 243

6 Perspectives 244

References 244

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1 Introduction

In the past 10 years, our understanding of the mechanisms of intracellular protein transport has dramatically increased. One of the most important achievements was the discovery of heat shock proteins (hsps) as modulators of protein folding. Since then, "chaperone mediated protein folding" has emerged as one of the most vivid areas of molecular cell biology. In this review, we will try to summarize our current knowledge of the function of molecular chaperones in intracellular protein traffic. It will become evident that in living cells the translocation of preproteins across membranes is accompanied at all stages by the interaction with a complex cellular protein-folding machinery.

Traditionally, molecular chaperones are defined as "a family of unrelated classes of proteins that mediate the correct assembly of other polypeptides, but are not themselves components of the final functional structure" (Ellis and van der Vies 1991). Molecular chaperones interfere with the three-dimensional structure of substrate proteins. They bind to and stabilize otherwise unstable conformers of other proteins and, by controlled binding and release of the substrate proteins, facilitate their correct fate in vivo (Hendrick and Hartl 1993). According to this definition, some enzymes are excluded from the group of molecular chaperones although they participate in protein-folding reactions. Such enzymes are the protein disulfide isomerases (PDIs). They catalyze covalent modifications which are of importance for the entire structure of the substrate protein. But in contrast to molecular chaperones, which stabilize the conformation of their substrates by the formation of complexes, these enzymes release their substrates very quickly. Protein-folding enzymes and molecular chaperones together constitute the protein-folding machinery of the cell. For a long time, many molecular chaperones were primarily known as "heat shock proteins" or "stress response proteins". These terms refer to the enhanced gene expression of many of these proteins under different forms of stress (reviewed in Lindquist and Craig 1988; Georgopoulos et al. 1991). However, most hsps are also constitutively expressed at considerable levels. In this review, we will focus on functional aspects of molecular chaperones under normal growth conditions.

Many proteins are synthesized in locations within the cell which are not their functional locations. This is the case for all proteins which are exported from the cell and for nearly all proteins of cell organelles. The biogenesis of organelles as well as the export of proteins involves the

synthesis of preproteins in the cytosol and their subsequent translocation across a biological membrane. Intracellular protein translocation across membranes requires protein-folding reactions in at least three steps (Fig. 1):

1. All preproteins which are synthesized prior to transport across membranes have to fold in a way which ensures a soluble and translocation-competent state.
2. The translocation itself requires the release of bound cytosolic cofactors and unfolding of the polypeptide in transit.
3. After translocation the imported proteins have to fold into their final and functional conformation.

The folding reactions at the ribosome (step 1) and the folding reactions in the lumen of the organelle (step 3) both start with an unfolded

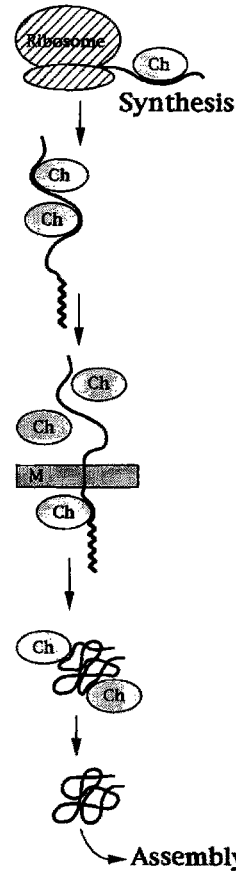

Fig. 1a. Translocation competence. Several cytosolic factors bind to nascent chains and confer a soluble and translocation-competent state. In this stage, molecular chaperones prevent the folding of preproteins

Fig. 1b. Translocation. Translocation across membranes (*M*) requires release of bound cytosolic factors and unfolding of the polypeptide in transit. In mitochondria and in the endoplasmic reticulum of yeast, translocation is driven by interaction with hsp70 in the lumen of the organelle

Fig. 1c. Folding. After translocation, folding is mediated by molecular chaperones, together with protein-folding enzymes and protein-modification enzymes

Fig. 1a–c. Pathway of a protein from synthesis to assembly; (*Ch*, chaperone protein)

protein, and both reactions are directed by a similar system of chaperone proteins. In eukaryotic cells, both systems apply homologs of the prokaryotic DnaK, DnaJ, GrpE family. (DnaK and DnaJ were, respectively, first described as components involved in the replication of DNA in *Escherichia coli*; Grp designated a glucose-regulated protein.) In functional terms, the two systems are separated by the unfolding of the substrate protein during translocation (step 2). The data obtained with mitochondria suggest that the unfolding of proteins in step 2 is mainly an indirect effect of the interaction with chaperone proteins in the lumen of the organelle. Up to now, no "unfoldase" on the surface of a eukaryotic membrane has been found. As we will describe in detail in later chapters, in some cases the interaction with homologs of the DnaK, DnaJ, GrpE family is followed by folding reactions which require a second chaperone system, a complex of homologs of the GroEL/ES system. (GroEL and GroEL are proteins that were identified by a gromutant of *E. coli*.Gro mutants are blocked in the development of bacteriophage λ at stages after adsorption and injection.)

The following chapter will give an introduction into the major systems of molecular chaperones which play a role in the translocation of proteins across membranes, and we will give a brief description of non-chaperone protein-folding enzymes.

2 Major Classes of Proteins Involved in Protein-Folding Reactions

2.1 The DnaK, DnaJ, GrpE System

Research on molecular chaperones of eukaryotic cells received considerable support from the comparison with homologous procaryotic components. It is now generally assumed that many reactions which previously were shown to be dependent on eukaryotic hsp70 (i.e., a heat shock protein of about 70 kDa), actually are dependent on a cooperation of at least three components, similar to DnaK, DnaJ and GrpE in *E. coli*. All three proteins are encoded by the same operon. DnaK is the only hsp70 protein of *E. coli*. Also, DnaJ and GrpE are encoded by single copy genes (Georgopoulos et al. 1991). *Saccharomyces cerevisiae*, on the other hand, contains eight different hsp70 genes and at least four homologs of DnaJ, reflecting the higher degree of complexity of eukaryotic cells (Craig and Lindquist 1988; Craig et al. 1993).

A mitochondrial homolog of GrpE has recently been identified in yeast (Ikeda et al. 1994; Laloraya et al. 1994; Voos et al. 1994).

Hsp70 molecules contain two domains. The aminoterminal domain of about 45 kDa has been shown to be an adenosine triphosphatase (ATPase) (Flaherty et al. 1990; Bork et al. 1992; Gao et al. 1994), the carboxyterminal domain is the substrate-binding domain (Liberek et al. 1991a). An 18-kDa fragment located immediately after the ATPase domain is sufficient for high-affinity binding (Wang et al. 1993). The substrate specificity is still a matter of debate. The high affinity of various hsp70s to denatured proteins led to the early hypothesis that the structures which are recognized are hydrophobic stretches of a polypeptide which are exposed upon denaturation (Pelham 1986). This view is in agreement with the results of an analysis of peptides which were eluted from BiP, the mammalian hsp70 of the endoplasmic reticulum (ER). (BiP was found as a binding protein for immunoglobulin heavy chains.) The released peptides contained preferentially hydrophobic amino acids (Flynn et al. 1991). The ATPase of hsp70 is not only stimulated by peptides (Flynn et al. 1989; Blond-Elguindi et al. 1993a), but also by monomeric amino acids at higher concentrations. Hydrophobic amino acids show the strongest stimulation (Richarme and Kohiyama 1993). In this line of evidence, it is a property of the primary structure which is recognized by hsp70. On the other hand, it has also been shown that not only hydrophobic, but also hydrophilic peptides can bind to BiP (Flynn et al. 1989). In fact, the binding specificity of hsp70 could be governed by a fundamentally different principle. This is suggested by nuclear magnetic resonance (NMR) studies of bound peptides. The results indicate an extended structure of the substrate (Landry et al. 1992). It can be assumed that it is not primarily the hydrophobicity of the substrate, but the lack of secondary structure which is the prerequisite for binding. This model is supported by the observation that reduced carboxymethylated α-lactalbumin, a substrate protein of little secondary structure, is bound by DnaK and also by mammalian hsp70 (Palleros et al. 1991). Additional support is provided by the report that the carboxy-terminal domain of a consensus secondary structure of different hsp70s can be modelled onto the crystallographic structure of the antigen-binding domain of a human leukocyte antigen (HLA) protein (major histocompatibility complex, MHC, class I) (Rippmann et al. 1991). These proteins have been shown to bind extended nine-amino-acid-residue peptides (Fremont et al. 1992; Matsumura et al. 1992) with considerable variation in the orientation of the side chains in the central part of the peptides (Madden et al. 1993). BiP seems to

preferentially bind segments of seven amino acids (Flynn et al. 1991). A screening of libraries of bacteriophages that display random peptides at their adsorption protein again selected hydrophobic peptides as high-affinity ligands of BiP (Blond-Elguindi et al. 1993b). Interestingly, most of these peptides contained aromatic and hydrophobic amino acids in alternating positions. It was suggested that the peptides were bound by BiP in an extended conformation with side chains of alternating residues pointing into a cleft on the BiP molecule.

There is now good evidence that hsp70 associates with newly synthesized proteins at the ribosome (Beckmann et al. 1990; Nelson et al. 1992; Morimoto 1993). The consequence of the binding of hsp70 seems to be that the extended structure of the growing chain is retained during synthesis. Consequently, the first effect of binding to hsp70 is not folding, but inhibition of folding of the substrate. The unfolded state of the substrate can be monitored by its extreme sensitivity against proteolytic degradation. Release of the substrate requires the presence of magnesium adenosine triphosphate (MgATP). In the presence of the chelator ethylene diamine tetra-acetic acid (EDTA), and in the presence of adenosine diphosphate (ADP) the association of a polypeptide with hsp70 is prolonged (Liberek et al. 1991a,b; Palleros et al. 1991; Langer et al. 1992a,b).

The differences in the affinity of hsp70s for substrate proteins in the presence of different nucleotides is not only of theoretical importance but also of practical interest for many biochemists. The stability of hsp70 complexes has been studied in a systematic investigation of DNA-K from *E. coli*, bovine brain hsp73 and human hsp72 with reduced carboxymethylated α-lactalbumin (Palleros et al. 1993). The data indicate that MgATP binding, but not ATP hydrolysis is essential for substrate dissociation. The reactions of hsp70 are also significantly influenced by potassium ions. Only the combination of K^+ and MgATP causes the conformational change in hsp70 that is necessary for substrate dissociation. In the absence of K^+, MgATP favors complex formation. Sodium ions cannot substitute for potassium ions. The rate limiting step for ATP hydrolysis seems to be the release of ADP and inorganic phosphate (Schmid et al. 1994).

In the absence of additional components, the ATP concentration present in the cytosol would cause a quick release of bound hsp70. In *E. coli* this release is prevented by DnaJ, a protein of 43 kDa (Langer et al. 1992a; Gamer et al. 1992; Wickner et al. 1992). In all cells, the concentrations of hsp70s seem to be similar to the concentrations of the corresponding DnaJ components. Under normal growth conditions, each of the two proteins comprises about 1% of the total cell protein.

The binding of DnaJ to hsp70 and to the substrate causes a two-fold stimulation of the hsp70 ATPase (Liberek et al. 1991b). In contrast to the hsp70-ATP substrate complex, the DnaJ-hsp70-ADP substrate complex is stable. Partial release of hsp70 could initiate the first major folding reaction of the substrate protein, the so-called hydrophobic collapse. One aspect of the function of hsp70 seems to be the delay of this collapse. Proteins which are bound to the DnaJ-hsp70 complex resemble folding intermediates of the "molten globules" type which accumulate as a result of the hydrophobic collapse (Bychkova et al. 1988; Ewbank and Creighton 1991; reviewed by Ptitsyn 1992; Creighton 1992; Matthews 1993). In this respect, it is justified to regard the creation of a molten globule in a test tube (by dilution of a urea-denatured protein into an appropriate buffer) as a model for the folding of substrate proteins at the surface of hsp70-DnaJ in the cell.

The release of the bound substrate from a DnaJ-DnaK-ADP complex is the result of an interaction with GrpE, a protein of 24 kDa (Langer et al. 1992a). GrpE stimulates the hydrolysis of ATP by the complex up to 50-fold, probably by an increase of the exchange of ADP against ATP. After release, some substrate proteins were found to show native folding. Apparently in these cases, the DnaJ, DnaK, GrpE system is sufficient to mediate a complete folding pathway. Other substrate proteins were characterized as folding intermediates which could be transferred to the GroEL/ES system. A sequential action of two chaperone systems was found in mitochondria (Kang et al. 1990; Manning-Krieg et al. 1991). The import pathway of some mitochondrial precursor proteins involves the transfer from mitochondrial hsp70 to hsp60, the mitochondrial homolog of GroEL.

2.2 Chaperonins and TCP1

The subclass of molecular chaperones which has been termed chaperonins is defined by subunits of about 60 kDa which assemble in 14-subunit oligomers of characteristic double toroid shape. The sequences of chaperonins are highly conserved. Members of this group are the hsp60 of the mitochondrial matrix, the rubisco-binding protein of the chloroplast stroma and the bacterial GroEL. All these proteins cooperate with co-chaperonins which form 7-subunit oligomers of 10 kDa subunit size. The t-complex polypeptide 1 (TCP1) complexes of eukaryotic cytosol show several similarities to chaperonins, but are regarded as members of a distinct subclass.

The most thoroughly investigated chaperonin is the GroEL of *E. coli* (Georgopoulos and Welch 1993; Martin and Hartl 1993). In the electron microscope, GroEL looks like a cylinder of 16 nm length with an outer diameter of 14.5 nm and an inner cavity of 6 nm diameter. This inner cavity is the substrate binding site (Langer et al. 1992b). As shown by electron microscopy, substrate proteins of up to about 90 kDa can be placed within the chaperonin. Alternatively, two smaller proteins, for example, two molecules of unfolded rhodanese (32 kDa) can bind to GroEL (Bochkareva et al. 1992). Gold-labeled dihydrofolate reductase (DHFR) was found to bind within individual rings of GroEL complexes (Braig et al. 1993). Substrates are accessible for different proteases and easily digested. This is interpreted to reflect their partly unfolded state.

Several observations suggest that the conformation in which substrate proteins are bound to GroEL is principally different from binding to hsp70. The same peptide which was detected in an extended structure when bound to DnaK, showed an α-helical conformation when bound to GroEL (Landry et al. 1992). Polypeptides of extended structure, which are excellent substrates for DnaK, DnaJ, GrpE chaperones, do not bind to GroEL (Langer et al. 1992a). These observations have prompted the assumption that chaperonins interfere with substrate proteins in a later step of the folding pathway than does hsp70. The hsp70 system could mediate folding of newly synthesized proteins until they have adopted a molten globule state; in this state substrates can be transferred to the GroEL system. It was demonstrated that substrates bound to GroEL show features of molten globules (Martin et al. 1991a; van der Vies et al. 1992; Ptitsyn 1992). However, recent data on the structure of GroEL-bound cyclophilin are in conflict with a general molten globule model. NMR investigations indicate that the complete secondary structure of cyclophilin is disrupted when bound to GroEL (Zahn et al. 1994). Clearly more experiments are needed to get a clear picture of the principles which determine the folding state of bound substrate proteins.

In the presence of MgATP, substrate proteins are folded by GroEL until they acquire their native conformation (Bochkareva et al. 1988, 1992; Goloubinoff et al. 1989b; Lissin et al. 1990; Brandsch et al. 1992). The reaction is stimulated by potassium ions (Viitanen et al. 1990). The folding of one molecule of DHFR has been measured to require the hydrolysis of about 130 molecules of ATP. The ATPase activity of GroEL is regulated by the co-chaperonin GroES, which can bind to GroEL (Martin et al. 1991a; Viitanen et al. 1991; Todd et al. 1993). It was shown that the two ends of a GroEL cylinder are equivalent until GroES

binds to one of them. The binding of a GroES oligomer to one end prevents the binding of a second GroES oligomer to the other end (Langer et al. 1992b; Burns et al. 1992). Isolated GroEL is only a weak ATPase and its activity is further repressed in the presence of stociometrically bound GroES. The GroES stabilizes GroEL in an ADP-bound state (Martin et al. 1993a). Binding of a substrate protein has three effects: (1) The ATPase activity of GroEL is drastically enhanced; (2) it triggers the release of ADP from GroEL; and (3) it causes the release of GroES. Upon ADP–ATP exchange, GroES reassociates. After hydrolysis of the ATP, the bound protein is released. GroES coordinates the activity of the 14 subunits in the chaperonin complex and allows coordinated binding and release of the substrate protein within the central cavity. Directed by GroES, all subunits of an individual ring proceed through the same states in parallel. Several cycles of these reactions are necessary to achieve complete folding, as only partially folded substrate proteins immediately rebind. Consequently, the GroEL/ES reaction cycle involves repeated binding and release of the substrate and of GroES. Within this cycle, GroES can bind to alternate sides of the GroEL toroid (Martin et al. 1993a; Martin and Hartl 1993). Interestingly, GroES itself binds ATP with an affinity similar to the affinity by which GroEL binds ATP (Martin et al. 1993b). It was speculated that ATP binding to GroES may be important in charging the seven subunits of the interacting GroEL ring with ATP.

TCP ring complexes are structures of about 970 kDa which have been found in eukaryotic cytosol. In the electron microscope, they look very similar to members of the chaperonin group. They form double toroid-shaped oligomers, with subunits of about 60 kDa. But in contrast to the sevenfold symmetry of chaperonins, TCP-oligomers show an eightfold symmetry. TCP-1 was the first protein of this family whose sequence was published (Gupta 1990). It shows sequence similarity to thermophilic factor (TF-55), a protein of the archaebacterium *Sulfolobus shibatae*, but only very limited similarity to chaperonins (Trent et al. 1991; Phipps et al. 1993). Nevertheless, TCP complexes can act in a chaperonin-like manner. The functional characterization of TCP complexes revealed ATP-dependent folding reactions comparable to GroEL (Lewis et al. 1992; Gao et al. 1992; Yaffe et al. 1992; Frydman et al. 1992; Mummert et al. 1993). In comparison to chaperonins, a distinct feature of TCP complexes seems to be that they lack the need for an analog to the co-chaperonins. The important question of whether the TCP complexes play an as general role in the eukaryotic cytosol as the chaperonins do in prokaryotes, chloroplasts and mitochondria, has not

yet received a satisfactory answer. Recent data demonstrate an involvement in the biogenesis of tubulin and actin in vivo (Ursic and Culbertson 1991; Sternlicht et al. 1993; Melki et al. 1993). The existence of at least seven different TCP-related genes in the mouse could indicate a diversity in the substrate specificity of individual TCP-complexes (Kubota et al. 1994). An involvement in intracellular protein traffic can be proposed, but experimental evidence is still lacking.

2.3 Hsp90

Hsp90 is one of the most abundant hsps of the cell. It is present in both the ER and cytoplasm under normal growth temperatures and accounts for 1%–2% of the total cytosolic protein. In mammals and in *S. cerevisiae*, hsp90 is encoded by two separate genes. Its products mainly exist as homodimers. Only recently hsp90 was shown to function as an ATP-independent molecular chaperone (Wiech et al. 1992, 1993; Melnick et al. 1992). However, the nature of the hsp90 interaction with substrate proteins is not understood. Several observations suggest regulatory functions of hsp90. Ca^{2+}-calmodulin binds to hsp90 and thereby inhibits the binding of hsp90 to actin filaments (Nishida et al. 1986; Minami et al. 1993). Also steroid hormone receptors and the oncogene product $pp60^{v\text{-}src}$ bind hsp90. The tyrosine kinase activity of $pp60^{v\text{-}src}$ bound to hsp90 is inhibited. Steroid hormone receptors lose their affinity for DNA by binding to hsp90, but show an increased affinity for the hormone. With respect to intracellular protein transport, it is noteworthy that hsp90 plays a role in the regulation of the location of the steroid hormone receptors in the cell. The chaperone-bound receptors are located in the cytosol, and the release of the chaperone allows transport into the nucleus and binding to the chromosomal DNA. In striking similarity to its role in the translocation of steroid hormone receptors, hsp90 is also involved in the partition of the dioxin receptor between cytosol and nucleus. The dioxin receptor (Ah receptor) is related in sequence to the steroid hormone receptors and plays a central role in the regulation of genes which are necessary in the phase I reactions of toxin elimination. Via the dioxin receptor hsp90 is, for example, involved in the regulation of the cytochrome P_{450}gene (Landers and Bunce 1991).

2.4 Hsp104

Hsp104 has been cloned in *S. cerevisiae* (Sanchez and Lindquist 1990). It forms homohexameric ring-shaped particles. Each subunit contains two ATP-binding sites, one of which appears to be involved in oligomerization (Parsell et al. 1994). Hsp104 is strongly heat inducible. Under normal growth conditions it is expressed only at very low levels. At temperatures between 25 and 37°C, hsp104 deletion mutants grow at the same rate as wild-type cells. At sublethal temperatures (37°C), yeast cells acquire an induced thermotolerance which seems to depend on the enhanced expression of hsp104. The effect of hsp104 can partially be substituted by hsp70 (Ssa1p), suggesting some similarity in the function of both proteins. Hsp104 is homologous to the ClpB protein of *E. coli*, which is part of an ATP-dependent protein degradation system. A mitochondrial homolog in yeast is hsp78 (Leonhardt et al. 1993).

2.5 Small Hsps

Small hsps have been investigated especially in plants, but have also been detected in vertebrates, drosophila and yeast. The mammalian representatives of this group form oligomeric structures of about 32 subunits and a total molecular weight of 800 kDa. The monomers are of 15–30 kDa (Arrigo et al. 1988; Behlke et al. 1991). Interestingly, α-B-crystallin, a protein of mammalian eye lenses, also belongs to this family. Small hsps seem to be molecular chaperones as well. They were shown to prevent aggregation and promote refolding of citrate synthase and α-glucosidase after urea denaturation in vitro. The reaction did not require ATP (Jakob et al. 1993). Up to now, the functional characterization did not reveal an involvement in protein translocation across membranes.

2.6 Peptidyl-Prolyl Isomerases

Experiments have shown that in vitro the last and very slow step in the folding pathways of many proteins is the isomerization of the amide-bond conformers of prolin residues. Due to its partial double-bond character, the peptide bond can adopt a *cis*- and a *trans* conformation. In equilibrium, the ratio of both conformers of proline in unfolded proteins is 1:3 (*cis:trans*). Protein-folding reactions reduce the content of *cis*-conformers to about 6% in native proteins. The half-time

of *cis–trans* isomerizations can be up to 20° min at 0°C, decreasing by a factor of about 3.3 for each 10 K rise in temperature (Brandts et al. 1975; Kiefhaber et al. 1990a,b; Texter et al. 1992). This reaction is catalyzed by peptidyl-prolyl *cis-trans* isomerases (PPIs), also called "immunophilins" (Fischer et al. 1984, 1989; Fischer and Bang 1985; Lang et al. 1987). The catalyzed reaction is a 180° rotation in the C-N linkage of the peptide bond preceding proline. PPIs comprise two families of proteins, namely cyclophilins and FK506 binding proteins (FKBPs) (recently reviewed by Schmid 1993). They are very abundant in all compartments of the cell, similar to hsp70. The sequences of cyclophilins and FKBPs are not related. Their prolyl isomerase activity can be inhibited by complex formation with cyclosporin A or FK506, respectively (Takahashi et al. 1989; Harding et al. 1989; Sierkierka et al. 1989; Breiman et al. 1992). Both reagents exhibit antibiotic activity. Strains of *Neurospora crassa* and of *S. cerevisiae* become resistant against cyclosporin A by loss of cyclophilin (Tropschug et al. 1989). The toxicity of cyclosporin A and of FKBP is not due to an inhibition of protein folding in the cell, since they are active in concentrations which only block a minor percentage of the cell's PPIs. Their complexes with PPIs interfere with signal transduction pathways of eukaryotic cells, and both inhibitors mediate pharmacological and toxic effects primarily by interfering with regulatory functions in the cell (Kunz and Hall 1993). The physiological significance of the prolyl isomerase activity of PPIs in vivo is far less obvious. Our knowledge of PPIs with respect to protein folding is mainly based on studies in vitro (Fischer and Schmid 1990).

Early investigations of the enzymatic mechanism of cyclophilin had suggested the involvement of a thiol group in the active center, since the activity of cyclophilin can be blocked by reagents directed against sulfhydryl groups (Fischer et al. 1989). However, it turned out that all cysteins of cyclophilin can be deleted without loss of activity (Liu et al. 1990). Current models of the mechanism of prolyl isomerization are mainly based on X-ray structures of complexes with substrate peptides. The mechanisms of cyclophilins and of FKBPs seem to follow different principles (Fischer et al. 1993). There is no experimental evidence of an influence of nucleotides on the activity of PPIs.

The primary structure of a given protein does not necessarily allow predictions of the effects of PPIs on its folding. The folding of some proteins is not catalyzed significantly, although good evidence exists that proline isomerization is important for their refolding (Lang et al. 1987). This is explained by an inaccessibility of PPIs to the respective residues

within partially structured folding intermediates. The activity of PPIs on the isomerization of short peptides is dependent on the amino acid preceding the proline and varies by a factor of 1000, depending on the PPI (cyclophilin or FKBP) and on the amino acid sequence of the substrate (Harrison and Stein 1990). PPIs have been classified as protein-folding enzymes in contrast to chaperone proteins. Only recently, it was suggested that in addition to their PPI activity, at least some of these proteins also have a chaperone function in protein-folding reactions. It was shown that the same cyclophilin can function in a late step of protein folding by acting as a prolyl isomerase, but also in an early step, by preventing the aggregation of a denatured substrate protein. Both activities are inhibited in the presence of cyclosporin A (Freskgard et al. 1992). Several PPIs turned out to be hsps or to be essential for viability at higher temperatures (Davis et al. 1992; Sykes et al. 1993; Partaledis and Berlin 1993). Apparently, molecular chaperones and protein-folding enzymes are not as different as had been assumed for a long time.

With respect to functions in vivo, the best characterized immunophilin is the Drosophila cyclophilin homolog NinaA (Shieh et al. 1989; Stamnes et al. 1991; Colley et al. 1991). NinaA is an integral membrane protein and required for the normal function of two different rhodopsins. Mutations in the NinaA gene severely inhibit opsin transport from the ER, leading to dramatic accumulations of ER cisternae in photoreceptor cells. Most mutations which were analyzed in a large screen for NinaA mutants were found in the peptidyl-prolyl substrate-binding region (Ondek et al. 1992). This observation is in agreement with the assumtion that the function of NinaA is related to its protein-folding activity. A more direct demonstration of the involvement of a PPI in protein-folding reactions in an organelle was recently provided by the analysis of a cyclophilin-deficient mutant of *N. crassa* (see Sect. 5.2).

2.7 Protein Disulfide Isomerases

PDIs have been found as soluble proteins of about 57 kDa in the lumen of the ER and in the periplasm of *E. coli*. PDI was the first protein that was suggested to be involved in the de novo folding of nascent polypeptide chains (De Lorenzo et al. 1966). It catalyzes the formation of disulfide bonds in proteins (Reviewed by Freedman 1989, 1992; Noiva and Lennarz 1992, Bardwell and Beckwith 1993). As disulfide bonds are preferentially found in secreted proteins, it is not surprising that in eukaryotic cells PDI is found only in a compartment of the secretory

pathway. It carries a carboxy-terminal sequence Lys-Asp-Glu-Leu (KDEL), which is characteristic for proteins resident in the ER.

In rat liver microsomes (ER vesicles), PDI amounts to about 2% of the entire protein content (Mills et al. 1983; Freedman 1989). It is part of different protein complexes:

1. PDI is identical with the β-subunit of prolyl 4-hydroxylase and thus involved in the posttranslational modification of procollagen (Koivu et al. 1987; Pihlajaniemi et al. 1987; Parkkonen et al. 1988). Surprisingly, the $\alpha_2\beta_2$ prolyl 4-hydroxylase holoenzyme is also active with enzymatically inactive PDI subunits. One of the functions of PDI in this complex seems to be the retention of the holoenzyme in the ER by its KDEL sequence (Vuori et al. 1992).
2. PDI has also been described as a component of the microsomal triglyceride transfer protein complex (Wetterau et al. 1990).
3. But not all of the PDI molecules in the ER are included in hetero-oligomeric enzyme complexes.

PDI also exists as a homodimer. PDI is identical with the "glycosylation site binding protein" of the ER (LaMantia et al. 1991) and is capable of binding peptides. The affinity for peptides seems to be independent of ATP. Cys-containing peptides bind four- to eightfold tighter than peptides of the same length lacking Cys. These and some other data led to the proposal that PDI is both an enzyme and a chaperone (Jaenicke 1993; Wang and Tsou 1993).

Investigations of the effect of PDI on the folding pathway of bovine pancreatic trypsin inhibitor revealed that PDI does not change the intermediates in the folding process but alters the kinetics of their appearance (Creighton et al. 1980). In the folding pathway of some proteins, PDI seems to be of crucial importance. γ-gliadin is unable to properly fold into its final, stable form in microsomes which have been depleted of PDI (Bulleid and Freedman 1988). In yeast, the gene for PDI is necessary for viability (Farquhar et al. 1991; LaMantia et al. 1991; LaMantia and Lennarz 1993).

3 Translocation Competence and Folding of Precursor Proteins in the Cytosol

3.1 Hsp70s and the Preservation of Transport Competence of Preproteins

The prepro-α-factor of yeast can be posttranslationally imported into microsomes in a cell-free system. Besides ATP this process requires the

Table 1. Major chaperone proteins in *E. coli* and their homologs in *S. cerevisiae* and mammals

	Prokaryotes	Eukaryotes	Yeast	Mammals
Hsp60 system (chaperonins)				
Hsp60 family	GroEl	Mitochondria	Cpn60 = Mif4p	Hsp58
Cooperating factor (co-chaperonin)	GroEs	Mitochondria	Cpn10	Hsp10 = Cpn10
TCP1 system	TF55 complex (Archaebact.)	Cytosol	Tcp1p	CCTα (= TCP1), CCTβ, CCTγ, CCTδ, CCTε, CCTη
Hsp70 system				
Hsp70 family	DnaK	Cytosol	Ssa1p, Ssa2p, Ssa3p, Ssa4p, Ssb1p, Ssb2p	Hsp70, Hsp72, Hsc73
		ER	Kar2p	BiP = Grp78
		Mitochondria	Ssc1p	Grp75
Cooperating with hsp70	DnaJ	Cytosol	Sis1p, Scj1p, Ydj1p = Mas5p	Hsj1, Hdj1, Hdj2, Hsp40
		ER	Sec63p	
		Mitochondria	Mdj1p	
	GrpE	Mitochondria	Yge1p = Mge1p	
Hsp90 family	HtpG	Cytosol	Hsc83, Hsp83	Hsp90α, Hsp90β
		ER		Grp94
Hsp110 family	ClpB	Cytosol/nucleus	Hsp104	Hsp110
		Mitochondria	Hsp78	

ER, endoplasmic reticulum; BiP, immunoglobulin heavy chain binding protein; chaperonins, chaperone proteins of the hsp60 type, comprising GroEL, the rubisco binding protein and mitochondrial hsp60; co-chaperonin, ring complexes of small subunits which cooperate with chaperonins GroES, cpn 10, and hsp 10; Cpn, chaperonin; Dnaj, J component involved in the replication of DNA in *E. coli*; DnaK, K component involved in the replication of DNA in *E. coli*; ER, endoplasmic reticulum; GroEL, protein which was identified by a gro mutant of *E. coli*. Gro mutants are blocked in the development of bacteriophage μ at stages after adsorption and injection; GroES, see GroEL; GRP, glucose-regulated protein; HDJ1, human homolog of DnaJ; Hsc73, hsp70 cognate protein of 73 kDa; HSjl, *Homo sapiens* homolog of DnaJ; Hsp, heat shock protein; Hspx, heat shock protein of about *x* kDa; Kar2, karyogamy mutant, Kar2p is the Kar2 protein; Mas5, mitochondrial assembly mutant; Mge1, mitochondrial homolog of GrpE; Mif4, mitochondrial import function; Rubisco-binding protein, binding protein of ribulose bisphosphate carboxylase in chloroplasts; SCJ1, *Saccharomyces cerevisiae* homolog of DnaJ; SEC, secretion mutant; SSA, stress protein of 70 kDa, subfamily A; TCP1, t-complex polypeptide 1; TF55, thermophilic factor 55; TRiC, TCP1 ring complex; YDJ1, yeast homolog of DnaJ; YGE1, yeast homolog of GrpE

presence of cytosolic factors (Hansen et al. 1986; Waters and Blobel 1986; Waters et al. 1986; Perara et al. 1986). The characterization of these factors led to the discovery of two distinct activities. One activity could be inhibited by the sulfhydryl reagent N-ethylmaleimide (NEM) and was suggested to be a protein. Its activity has also been described for reticulocyte lysate and for several different precursor proteins (Zimmermann et al. 1988; Randall and Shore 1989; Sheffield et al. 1990; Young et al. 1990). The NEM-sensitive factor itself has never been identified. The other translocation stimulating activity was NEM-resistant and could be purified. It consists of two 70 kDa hsps which were identified as the hsp70s Ssa1p and Ssa2p (Chirico et al. 1988; Murakami et al. 1988). Both proteins are constitutively expressed (in contrast to Ssa3p and Ssa4p, which are expressed in considerable amounts only under stress conditions). In a parallel approach, yeast mutants were characterized which had the chromosomal genes *SSA1, 2*, and *4* deleted and contained the *SSA1* gene under the control of an inducible promotor on a plasmid (Deshaies et al. 1988). This system allowed investigation of the effects of different expression levels of *SSA1* in vivo. The authors showed that low levels of Ssa1p caused an accumulation of prepro-α-factor and of the mitochondrial precursor protein F_1 β in the cytosol. More recently, a dependence on hsp70 and on an NEM-sensitive factor was also demonstrated for the import of proteins into the nucleus (Shi and Thomas 1992). Both genetic and biochemical experiments had thus shown a general importance of hsp70s for the transport of preproteins in the cell.

By which mechanism do the hsp70s exert their function? The functions of Ssa-proteins seem to be similar. Strains with either deletions of *SSA1, 2, 3* or *4* are viable and all four proteins seem to be able to substitute for each other (although differences were observed between the affinities of distinct SSA-proteins in processes such as uncoating of clathrin-coated vesicles (Gao et al. 1991)). It was discussed that hsp70 could interfere with translocation in several ways: It could keep the precursor proteins in a translocation-competent conformation, dissolve aggregates, unfold the precursor, or bind hydrophobic parts of presequences (Pelham 1986; Wiech et al. 1987; Meyer 1988; Rothman 1989). Several reports demonstrated that tightly folded preproteins do not traverse membranes (Eilers and Schatz 1986; Eilers et al. 1988; Vestweber and Schatz 1988a; b; Pfanner et al. 1988; Rassow et al. 1989; Wienhues et al. 1991). In the past few years, the interpretation of the effects of hsp70 in eukaryotic cytosol has been stimulated especially by the results obtained by investigations of the hsp70 system of *E. coli*. As described

in Sect. 2.1, hsp70 is thought to bind to nascent chains at the ribosome, keeping bound polypeptide chains in an extended conformation and thereby delaying further folding reactions. This process does not entail the dissolving of aggregates, but it keeps hydrophobic proteins soluble and prevents tight folding of the substrate proteins. Both aspects together seem to constitute the translocation-competence of precursor proteins.

3.2 DnaJ Homologs

On the basis of the comparison with the hsp70 system of *E. coli*, it was postulated that also in eukaryotes hsp70 should not act alone but in cooperation with other chaperone proteins, i.e., homologs of DnaJ and GrpE. In fact, it has already been known for a long time that in the cytosol precursor proteins are contained within high molecular weight complexes of about 250 kDa (Ohta and Schatz 1984; Ono and Tuboi 1988). In yeast, several DnaJ homologs have been identified (Silver and Way 1993). One of these is YDJ1 (the mitochondrial assembly mutant MAS5), which was identified in two independent genetic screens (Atencio and Yaffe 1992; Caplan and Douglas 1991). Deletion mutants of this gene grow slowly at 23°C and are inviable at 37°C. The protein was localized in the cytosol and shown to be involved in the intracellular transport of prepro-α-factor and of mitochondrial precursor proteins. By a carboxy-terminal farnesyl moiety it is bound to membranes, especially to the nuclear envelope. The farnesylation is required for the function of the Ydj1 protein at elevated temperatures (Caplan and Douglas 1991; Caplan et al. 1992b).

Investigations of Ydj1p have improved our understanding of the molecular mechanisms of "translocation-competence" in the eukaryotic cytosol. The function of Ydj1p was investigated in vitro in a system containing the purified components Ssa1p (cytosolic hsp70), the permanently unfolded protein carboxymethylated α-lactalbumin, Ydj1p and MgATP (Cyr et al. 1992). The Ydj1 protein used in this study was not farnesylated. With this system it was found that complexes of α-lactalbumin and Ssa1p are stable in the presence of MgATP. Complexes between α-lactalbumin and Ydj1p were not observed. The release of the bound substrate by Ssa1p was dependent on the hydrolysis of the bound ATP. The rate of hydrolysis of ATP by Ssa1p was stimulated by the addition of Ydj1p about tenfold. Maximal stimulation of Ssa1p ATPase activity was observed at a Ydj1p:Ssa1p molar ratio near 1. Ydj1p

Fig. 2a. Hsp70 (Ssa1p) and Ydj1p of *S. cerevisiae* (Ydj1p is membrane bound;modified after Cyr et al. 1992).*From top to bottom*: Stable complex of substrate with hsp70 + ATP. Release of the substrate triggered by interaction with Ydj1p, followed by hydrolysis of ATP

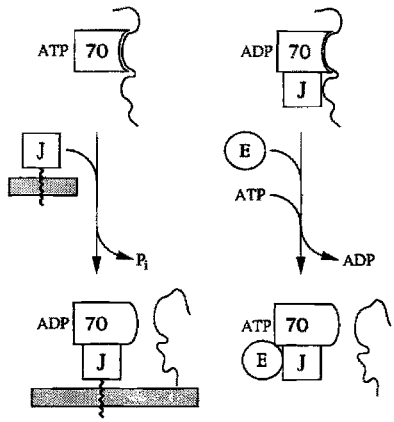

Fig. 2b. Hsp70 (DnaK), DnaJ and GrpE of *E. coli* (DnaJ is soluble; modified after Langer et al. 1992a) Stable complex of substrate with hsp70 + DnaJ + ADP. Release of the substrate triggered by interaction with GrpE, followed by exchange of ADP for ATP

Fig. 2a,b. Function of the homologous proteins Ydj1p and DnaJ in cooperation with hsp70

apparently stimulates the ATP-dependent release of the substrate prebound to Ssa1p (Fig. 2). On the basis of these data, it can be assumed that in the cytosol preproteins are kept translocation-competent by an association to hsp70 proteins and are sufficiently stable in these complexes in the presence of physiological concentrations of ATP. As soon as these complexes reach a target membrane, they can associate with Ydj1p which is concentrated at membranes due to its farnesyl moiety. Ydj1p then stimulates the hydrolysis of hsp70-bound ATP and by doing so initiates the release of the preprotein from the complex. The released preprotein is transferred to the import machinery of the target membrane in a translocation-competent conformation. The farnesyl moiety of Ydj1p guarantees that the release of the preprotein is only induced at the membrane and that the stability of the preprotein complex is not jeopardized in the cytosol.

It would be interesting to test whether the other known eukaryotic DnaJ proteins behave similar to Ydj1p. Only recently, it was shown that the plant molecular chaperone Anj1p is farnesylated and associated with microsomes (Zhu et al. 1993). In yeast, three further DnaJ homologs are known: the essential genes *SIS 1* and *SEC 63* and the nonessential gene *SCJ 1*. Sis1p was found in the cytosol and in the cell nucleus (Luke et al. 1991), Sec63p is a membrane protein of the ER (Sadler et al. 1989; Rothblatt et al. 1989), and Scj1p possibly is a soluble protein in the same compartment (Blumberg and Silver 1991). Three human DnaJ homologs have also been cloned (Raabe and Manley 1991; Cheetham et al. 1992; Ohtsuka 1993). The only other extensively investigated J protein is the prokaryotic DnaJ protein (Zylicz et al. 1989; Straus et al.

1990; Georgopoulos et al. 1991; Liberek et al. 1991b; Wickner et al. 1991; Gamer et al. 1992; Gragerov et al. 1992; Wickner et al. 1992; Langer et al. 1992a; Hendrick et al. 1993). It is noteworthy that substrate complexes with hsp70 and DnaJ of *E. coli* were found to be quite stable, in contrast to the corresponding complex with Ydj1p. The DnaJ protein was proposed to be a stabilizing factor of the *E. coli* chaperone system (Langer et al. 1992a), while Ydj1p was suggested to be a release factor of the chaperone system in yeast (Cyr et al. 1992). In *E. coli*, the ATP-dependent release of substrate proteins from hsp70 seems to be stimulated not by the DnaJ protein but by a third component, the GrpE protein (Fig. 2). Future research will hopefully reveal whether the different eukaryotic DnaJ proteins also show different functions and mechanisms in the cell.

3.3 The ATP Requirement of Translocation-Competent Preproteins in the Cytosol

The import of preproteins into cell organelles requires ATP in different steps of translocation. To discern the role of ATP in the successive steps of translocation, in vitro assay systems were depleted from ATP and the accumulated translocation intermediates were characterized. We have already seen that the translocation-competence of preproteins involves the activity of hsp70s, which is dependent on ATP. Therefore, it should be expected that the translocation-competence of preproteins in the cytosol depends on the concentration of ATP. In fact, such effects have been reported, but they are not necessarily always due to an involvement of hsp70.

Protein import into chloroplasts has been reported to need ATP outside the membranes (Grossman et al. 1980, Flügge and Hinz 1986). A closer investigation showed that the hydrolysis of ATP is needed for high-affinity binding of precursor proteins to the outer surface of chloroplasts. The concentration of ATP that is needed for maximum stimulation of binding is approximatly one-tenth that needed for translocation (Olsen and Keegstra 1992). Unfortunately, the precise role of ATP in protein binding is still unknown. Currently it is assumed that this ATP is not required in the cytosol but in the intermembrane space, and possibly related to protein kinases which have been identified in the chloroplast envelope membranes (Hinz and Flügge 1988; Soll and Bennett 1988).

Similarly, the import of preproteins into peroxisomes requires ATP (Imanaka et al. 1987; Wendland and Subramani 1993). *PAS1*, one of

the first cloned genes which are required for peroxisome biogenesis, turned out to encode a putative ATPase of about 117 kDa (Erdmann et al. 1991). The hydropathy profile of the predicted Pas1 protein does not show any satisfactory evidence for a membrane-spanning region, but indicates a rather hydrophilic protein. Due to its very low abundancy, it has been impossible up to now to localize the protein in the cell. There is no experimental evidence that the Pas1 protein acts as an ATP-dependent molecular chaperone. As with the import into chloroplasts, the ATP dependence of protein import into peroxisomes could not be directly attributed to the cytosolic chaperone system.

The role of cytosolic ATP in protein translocation was investigated at distinct steps of the import of the ADP/ATP carrier into mitochondria (Pfanner et al. 1987, 1988; Pfanner and Neupert 1987). In an in vitro system containing reticulocyte lysate and isolated mitochondria, the first of these steps, high-affinity binding to an import receptor at the outer surface of the organelle, showed only a minor dependence on ATP. The mitochondrial import receptor has been identified: the ADP/ATP carrier binds to the 72-kDa component MOM72 of the mitochondrial import receptor complex (Pfanner et al. 1991). The receptor-bound preprotein is very sensitive against digestion by proteases, indicating a high degree of unfolding. Probably the preprotein is kept in a loosely folded form by chaperone proteins of the cytosol and then transferred to the receptor in a reaction which involves the hydrolysis of ATP due to partial release of hsp70. The next step, release from the receptor and translocation across the outer membrane, requires higher concentrations of ATP, which may be necessary for complete release of cytosolic factors.

If the ATP dependence of protein import in the cytosol is due to the involvement of chaperone proteins, import in the absence of such cofactors should be independent of ATP. This was tested by import of purified precursor proteins. They were dissolved in 8 *M* urea and diluted into the import assay. Targeting of a chemically pure preprotein to mitochondria requires an import receptor, but it does not require the addition of a cytosolic chaperone protein or signal recognition factor (Becker et al. 1992). And, in fact, the import of some proteins, for example, the mitochondrial outer membrane protein porin, does not need ATP after unfolding by 8 *M* urea (Pfanner et al. 1988). Furthermore, shortening of precursor proteins should diminish the number of bound chaperone proteins and thus reduce the need for ATP in their release. This was shown for a series of fusion proteins of an amino-terminal part of cytochrome b_2 (mitochondrial lactate dehydrogenase) and DHFR

(Rassow et al. 1989; Pfanner et al. 1990; Koll et al. 1992). Effects of ATP which are probably due to the release of cytosolic chaperone proteins have been reported for F_1 β, CoxIV-DHFR, cytochrome c_1 and other preproteins (Pfanner and Neupert 1986; Pfanner et al. 1987; Chen and Douglas 1987b; Eilers et al. 1987, 1988; Pon et al. 1989). The effects of nucleotides in import into the ER will be considered in the following chapter.

3.4 Chaperone Activities in Targeting

Targeting is a major aspect of translocation competence. In the first parts of this chapter we did not consider this aspect because we first wanted to describe the general features of preproteins. It is now generally assumed that hsp70 plays a very general role in the import of precursor proteins of many different organelles. And DnaJ homologs probably are of general importance for the regulation of the hsp70 ATPase. We have seen that these proteins, probably together with other chaperone proteins, keep the precursor proteins soluble and in a conformation which is compatible with translocation across the target membranes. But only by specific targeting mechanisms can the different preproteins be delivered to their appropriate target membranes. This is mainly accomplished by characteristic presequences of the precursor proteins (Hurt et al. 1984; Horwich et al. 1985; von Heijne 1986; Roise and Schatz 1988; von Heijne et al. 1989; Hartl and Neupert 1990). Obviously, the chaperone proteins of the cytosol must bind to their substrate proteins in a way which allows the interaction of the presequences with their corresponding targets at the membranes. How is this achieved?

Specific signal sequence- or presequence-binding proteins have been identified, which are involved in targeting to the ER and to mitochondria. They are generally not regarded as chaperone proteins. However, their activity is in part similar to chaperone activities and their function is dependent on a balanced cooperation with the hsp70 system of the cytosol. Up to now, no data are available concerning the different affinities of presequence binding proteins and chaperone proteins to presequences. It is, however, evident that the function of the presequence binding factors (PBFs) is completely dependent on the ratio of their affinities and concentrations in relation to those of the different chaperone proteins. Two systems seem to exist in parallel. The components of the hsp70 system are of very high concentration but of relatively low affinity for the presequences. The presequence binding proteins are of

low concentration in the cytosol, but of high affinity for the corresponding presequences.

The best characterized targeting system of the cytosol is the signal recognition particle (SRP) and the SRP receptor of the ER membrane, comprising the two subunits SR$\alpha$and SRβ (Walter and Blobel 1981; Gilmore et al. 1982; Meyer et al. 1982; Sanz and Meyer 1988). Most ER preproteins which are longer than 80 amino acids interact with the SRP as soon as they appear at the ribosome exit site. The 54 kDa component of SRP (SRP54) binds to the amino-terminal signal sequence which is characteristic for ER preproteins (Kurzchalia et al. 1986). This association slows down the translation until the nascent chain reaches the ER target membrane. SRP binds to the SRP-receptor, initiating the binding of guanosine triphosphate (GTP) to the α-subunit of the recep-

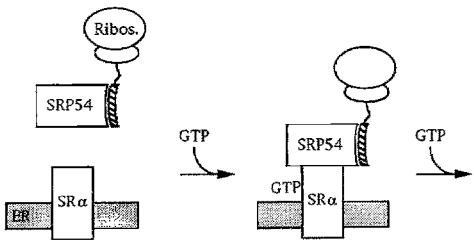

Fig. 3a. The targeting complex arrives at the ER membrane with SRP54 held in a nucleotide-free state. Binding to the SRP receptor stimulates GTP binding to the α-subunit of the SRP receptor and to SRP54 (*Ribos*, ribosome)

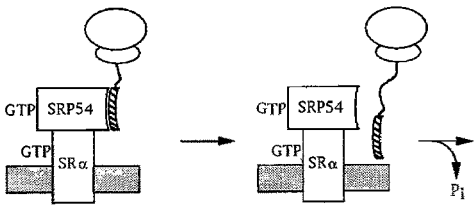

Fig. 3b. Binding of GTP to SRP54 causes the release of the bound signal sequence (*hatched*). The signal sequence can interact with the import apparatus of the ER

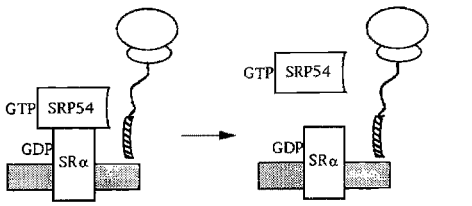

Fig. 3c. Hydrolysis of the SR α-bound GTP triggers the dissociation of SRP54 from its receptor. Hydrolysis of the SRP54-bound GTP precedes binding of a new signal sequence

Fig. 3a–c. Function of SRP54 and SRα in targeting of preproteins to the translocation pore of endoplasmic reticulum (*ER*) membranes. SRP54 is a subunit of the signal recognition particle (*SRP*), SRα is a subunit of the SRP receptor. Both proteins were isolated from canine pancreas rough microsomal membranes. (Modified after Miller et al. 1993)

tor. Interestingly, SRP54 also contains a GTP-binding domain and this "G domain" seems to be essential in this step (Zopf et al. 1993). The SRP receptor both increases the affinity of SRP54 for GTP and activates its GTPase (Miller et al. 1993). Binding of GTP to SRP causes the release of the signal sequence, the elongation arrest is released, and the signal sequence interacts with the import apparatus of the ER membrane. The cycle is completed by the dissociation of SRP from its receptor, triggered by the hydrolysis of the receptor bound GTP (Fig. 3) (Connolly and Gilmore 1989; reviewed in Rapoport 1990; Nunnari and Walter 1992; Sanders and Schekman 1992; Gilmore 1993). This cycle is essential for the import of proteins into the ER of mammalian cells. Possibly, SRP-independent preproteins are targeted to the ER by free SRP54 subunits (Zimmermann and Meyer 1986; Lutcke et al. 1992). In yeast, a homologous SRP system was identified but seems to be nonessential (Hann and Walter 1991). In this scheme the affinity and specificity of SRP54 is central for the function of the entire cycle. SRP54 recognizes the special features of the signal sequences and mediates and directs the traffic of the ER preproteins in the cytosol. In binding to the signal sequence SRP54 competes with the more unspecific chaperone proteins like hsp70. The scheme also provides an explanation for the dependence of the process on GTP. All three proteins, SRP54 and both subunits of the SRP receptor contain putative GTP-binding domains.

An additional nucleotide-dependent step has been identified in yeast: At the ER membrane the signal sequence is first transferred to the Sec62 protein and from there to the Sec61 protein. The transfer from Sec62p to Sec61p is ATP-dependent (Sanders et al. 1992; Müsch et al. 1992). A mammalian homolog of Sec61p has recently been identified (Görlich et al. 1992a), which is a further indication of similar mechanisms in both organisms. The mammalian Sec61p seems to be identical with the previously identified "imp34", a nonglycosylated protein of 34 kDa (Kellaris et al. 1991). In mammalian microsomes, Sec61p has been suggested to serve not only as part of the import channel but also as a ribosome receptor. The importance of the mammalian ER protein p180 as a ribosome receptor is still unclear (Görlich and Rapoport 1993; Savitz and Meyer 1993). Besides Sec61p, a glycoprotein of 54 kDa, named TRAM has been characterized as an additional major component of the mammalian ER import apparatus (Görlich et al. 1992b). The SSR-complex which was favored in this respect for a long time actually seems to be of minor importance in these reactions (Hartmann et al. 1993). Mammalian homologs of Sec62 and Sec63 have not been found. In reconstituted proteoliposomes, purified SRP receptor, Sec61p, and

TRAM are sufficient to establish translocation activity (Görlich and Rapoport 1993).

Short preproteins of less than 80 amino acids can be targeted to mammalian microsomes in the absence of signal recognition factors (Müller and Zimmermann 1987). Upon synthesis at the ribosome, they bind to cytosolic chaperone proteins, they are targeted to the ER by their presequences, and they are imported post-translationally. The requirements for the import of prepromellitin into microsomes were analyzed in detail. This protein seems to be targeted to the ER by an amino-terminal loop structure. For translocation across the membrane, this loop must be destabilized or unfolded. This step is catalyzed by a cytoplasmic protein and ATP. Depletion of ATP leads to inhibition of membrane insertion (Müller and Zimmermann 1988).

The characterization of factors which bind to the presequences of mitochondrial precursors still awaits the cloning and sequencing of the identified proteins. Two factors were purified from reticulocyte lysate: a "targeting factor" of 28 kDa (Ono and Tuboi 1990a,b) and a "presequence binding factor" (PBF) of 50 kDa (Murakami and Mori 1990). PBF was shown to bind to the precursor form of ornithine carbamoyltransferase but not to the mature protein. Binding was inhibited in the presence of synthetic presequences. The factor stimulates import into mitochondria. This effect is enhanced by added hsp70. The major obstacle on the way to further insights into the physiological role of these factors is probably their low abundancy in reticulocyte lysate which makes experiments difficult. Recently, a mitochondrial import stimulating factor (MSF) was isolated from rat liver cytosol (Hachiya et al. 1993). MSF is not a presequence binding factor but promises to be a true chaperone protein. Possibly it plays a role in the specificity of targeting of mitochondrial preproteins. MSF is composed of two polypeptides with molecular masses of 30 and 32 kDa. The heterodimer was reported to exhibit two activities, namely ATP-dependent prevention of aggregation of precursor proteins, and NEM-sensitive stimulation of import into mitochondria.

3.5 Import of Proteins into the Nucleus

Proteins are imported into the nucleus via pores in the nuclear membrane of about 9 nm diameter. Proteins of up to about 50 kDa can easily pass through these pores unless their transport is prevented by special mechanisms (Peters 1986). Their transport across the membrane does

not necessarily involve the unfolding of the polypeptide in transit. In vitro, signal sequence-coated gold particles of 5–20 nm diameter can be imported into nuclei (Feldherrr et al. 1984). The same pore can accommodate both RNA exit and protein import (Dworetzky and Feldherr 1988). The pore is constituted by an exceptionally large protein complex. The mass of this nuclear pore complex (NPC) has been determined to be approximately 120 MDa, which equals the mass of about 30 eukaryotic ribosomes (Reichelt et al. 1990). The association of the pore complex with the nuclear lamina has made it difficult to characterize its components. The currently known pore proteins have been estimated to account for less than 5% of the total NPC mass. One of the earliest steps in import of proteins through nuclear pores may be the association of the translocating proteins with a network of fibers that span the pore (Georgatos and Blobel 1987; Richardson et al. 1988). In some aspects, translocation across the nuclear membrane apparently applies completely different mechanisms than translocation across the more or less sealed membranes of other organelles. However, hsp70 is also involved in nuclear protein uptake (Imamoto et al. 1992; Shi and Thomas 1992).

Targeting of nuclear proteins is directed by nuclear localization sequences (NLS). These are short sequences of basic amino acids. In the case of the SV40 T antigen this sequence is Pro-Lys-Lys-Lys-Arg-Lys-Val (Kalderon et al. 1984). Some proteins do not possess their own NLS and enter the nucleus by cotransport with another protein. This principle gave the basis for a novel genetic system to detect protein-protein interactions using the GAL4 transcription factor (Fields and Song 1989). Several NLS binding proteins have been identified (reviewed in Silver 1991). Their functional location seems to be partly at the nuclear membrane, partly in the cytoplasm. In the latter case they are thought to first bind in the cytoplasm and then deliver the proteins to the pore complex. When nuclear import is inhibited by depletion of ATP, nuclear proteins accumulate in the cytoplasm or at the nuclear surface (Richardson et al. 1988; Breeuwer and Goldfarb 1990). Some of the NLS binding factors of mammalian cytosol are NEM-sensitive (Newmeyer and Forbes 1990). This finding is reminiscent of the NEM-sensitive factor of yeast cytosol described for the import into the ER and mitochondria (Waters et al. 1986). Examination of import into isolated nuclei from yeast did not show a similar inhibition by NEM (Kalinich and Douglas 1989). This again indicates that import into different organelles requires different sets of cofactors.

In the past few years, the involvement of immunophilins and hsp90 in nuclear import has received special attention. Several steroid

hormone receptors were found to bind to these proteins in their inactive state. In this context it became evident that nucleocytoplasmic transport of transcription factors are important in the regulation of gene expression. This was first shown for the glucocorticoid receptor (Sanchez et al. 1985; Picard and Yamamoto 1987; Picard et al. 1988, 1990; Bresnick et al. 1989; reviewed by Pratt 1993). In the absence of glucocorticoids, the receptor is localized in the cytosol. In the presence of the hormone it translocates into the nucleus and binds to DNA. The location of the glucocorticoid receptor within the cell is regulated by the accessibility of its NLS. The NLS is obscured as long as hsp90 is bound to the receptor. Hsp90 is released upon binding of the steroid hormone. Besides determining the location of its receptor the hormone also activates it. The activation of the hsp90-bound receptor is an ATP-requiring reaction (Hutchison et al. 1992; Smith et al. 1992; Pratt et al. 1992; Csermely et al. 1993). Interestingly, besides hsp90 the complex of the glucocorticoid receptor also contains the PPI FKBP59 (Yem et al. 1992; Tai et al. 1992; Peattie et al. 1992; Ruff et al. 1992; Tai et al. 1993). The function of the PPI in this complex is not understood. Folding and function of the receptor are not modified by binding of FK506 (Hutchison et al. 1993). Binding of a PPI is not restricted to the glucocorticoid receptor. The unactivated bovine estrogen receptor complex contains a FKBP59 and additionally an estrogen receptor binding cyclophilin (ERBC) of 40 kDa (Ratajczak et al. 1993). The inner architecture of the complex is not known. Probably hsp90 mediates the binding of the other components as immunophilins also bind to immobilized hsp90 (Nadeau et al. 1993).

Similar to the steroid hormone receptors, the mammalian transcription factor $NF_{\kappa}B$ contains a NLS and shuttles between cytosol and nucleus. Its regulation involves the protein $I_{\kappa}B$ (Baeuerle and Baltimore 1988). $I_{\kappa}B$ associates with $NF_{\kappa}B$ in the cytosol and prevents transport into the nucleus by masking the NLS (Zabel et al. 1993). Phosphorylation of $I_{\kappa}B$ disrupts the $I_{\kappa}B$-$NF_{\kappa}B$ complex and $NF_{\kappa}B$ enters the nucleus (Ghosh and Baltimore 1990). Nuclear localization of $NF_{\kappa}B$ is also induced by oxidative stress. $NF_{\kappa}B$ is a heterodimer of proteins of 50 and 65 kDa. They are homologous to each other and to the proto-oncogene c-*rel* and to the Drosophila morphogen dorsal (reviewed in Gilmore 1990). Two additional homologs have been identified recently. Nuclear uptake of these proteins is controlled by equivalents of $I_{\kappa}B$. This is the $I_{\kappa}B$ homolog pp40 for c-*rel* and the cactus protein for dorsal. Genetic evidence suggests that in Drosophila the cactus protein is regulated by the gene products of pelle, tube, and toll. This demon-

strates that in cytosolic protein traffic the more unspecific chaperone system is supplemented by a set of specific and highly regulated binding proteins.

4 Translocation of Preproteins Across Organelle Membranes

What drives proteins across membranes? In this section, we will discuss some models which have been developed to answer this question with respect to mitochondria, chloroplasts and the ER. We will especially concentrate on the mechanism of protein translocation across the mitochondrial membranes because, at the moment, mitochondria represent the best understood system. In mitochondria, the hsp70 of the matrix compartment is obviously the component which drives translocation of preproteins across both mitochondrial membranes and which causes the ATP-dependence of the process. In other organelles the mechanism of protein translocation seems to be similar.

4.1 Mitochondria

Some of the very first experiments which were performed to investigate the import of proteins into mitochondria addressed the question of the energetics of the process. It was suggested that proteins are imported into mitochondria cotranslationally and that the driving force of translocation was mediated by the translation machinery of the ribosomes (Kellems and Butow 1972; Kellems et al. 1974). However, it was then shown in vivo and in vitro that import into mitochondria can occur post-translationally. Consequently, translation does not provide the driving force of translocation (Hallermeyer et al. 1977). We will now summarize our current knowledge of the energetics of protein import into mitochondria, considering the conformation of the preproteins, the role of the membrane potential and of ATP and the function of mitochondrial hsp70.

4.1.1 The Conformation of Preproteins During Translocation

As we have discussed in the previous sections, preproteins adopt a loosely folded conformation in the cytosol. This is a characteristic aspect of their translocation competence. How does this conformation change during the translocation across the mitochondrial membranes? The

observation that preproteins can be arrested in translocation sites with their amino terminus located in the matrix and their carboxy terminus exposed to the cytosol seems to exclude a translocation of these proteins in a compact conformation (Schleyer and Neupert 1985). Direct evidence for an unfolding reaction as a major prerequisite for translocation was provided by a landmark experiment using a fusion protein of DHFR fused to the presequence of the mitochondrial precursor protein cytochrome oxidase subunit IV (COX IV) (Eilers and Schatz 1986). This fusion protein can efficiently be imported into isolated mitochondria in vitro (Hurt et al. 1984). Strikingly, this protein is not imported in the presence of methotrexate, a specific inhibitor of DHFR that stabilizes the native conformation of the protein. Apparently, methotrexate induces a very tight folding of the DHFR which prevents an unfolding reaction. Authentic DHFR is a cytosolic protein which is released from the cytosolic protein-folding machinery as an enzymatically active monomer. The binding of methotrexate to the COX IV-DHFR fusion protein demonstrates that the DHFR domain is kept in its native conformation also in the fusion protein. The folding and unfolding reactions which are influenced by the methotrexate in the import assay are not related to interactions with the chaperone system of the cytosol but indicate a folding reaction which is solely related to the translocation across the mitochondrial membranes. This is confirmed by the fact that methotrexate is effective also in import assays with purified preproteins.

A similar system was established, applying a fusion protein which contained a metallothionein domain. In this case it was possible to prevent translocation by the addition of copper ions (Chen and Douglas 1987a).

A series of fusion proteins between a DHFR domain and amino-terminal parts of the mitochondrial precursor protein cytochrome b_2 was constructed to investigate to which degree a protein is unfolded during translocation. In the presence of methotrexate these fusion proteins accumulate in translocation sites with the entire DHFR domain staying outside the mitochondrial membranes while the presequence reaches into the mitochondrial matrix and is cleaved off by the matrix-localized processing peptidase (MPP; Kalousek et al. 1993; Rassow et al. 1989). Thereby the minimal number of amino acids necessary to span both mitochondrial membranes was determined. It was found that 50 amino acids are sufficient to span both membranes. This indicates that the polypeptide in transit is unfolded and probably adopts an extended conformation (Fig. 4) (Rassow et al. 1990). To span two lipid bilayers the

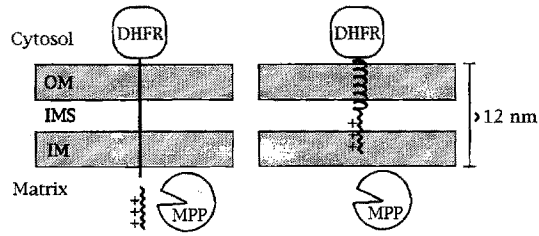

Fig. 4. The conformation of polypeptides in transit through the import sites of mitochondrial membranes. Experiments with cytochrome b_2-DHFR fusion proteins show that 50 amino acids are sufficient to span both membranes of mitochondrial translocation sites. It can be concluded that preproteins traverse the mitochondrial membranes in extended conformation. In α-helical conformation these peptides would not be of sufficient length to span both membranes and allow processing by MPP in the matrix (*OM*, mitochondrial outer membrane; *IM*, mitochondrial inner membrane; *IMS* intermembrane space; *MPP*, matrix-localized processing peptidase; *DHFR* dihydrofolate reductase. (After Rassow et al. 1990)

polypeptide has to span at least 12 nm; in α-helical conformation, a polypeptide of 50 amino acids could only span a distance of 7.5 nm. In extended conformation the same polypeptide can span 18 nm, which matches the actual distance that was measured at translocation contact sites in electron micrographs. The cytochrome b_2-DHFR fusion proteins which were used in these experiments are imported via the same translocation sites as authentic precursor proteins (Rassow et al. 1989). The extended conformation of proteins during translocation is likely to be a characteristic feature of precursor proteins when they approach the protein folding machinery of the mitochondrial matrix. In extended conformation preproteins should be an ideal substrate for hsp70.

4.1.2 Hsp70 and the Mechanism of Translocation

Before a preprotein encounters the components of the mitochondrial matrix, it has already interacted with the protein translocation machineries of the mitochondrial outer and inner membranes. Currently a model is favored (Fig. 5) which proposes the existence of independent transport machineries in outer and inner membranes (Glick et al. 1991; Rassow and Pfanner 1991; Jascur et al. 1992; Pfanner et al. 1992). Translocation of preproteins through the outer membrane is mediated by a complex of at least eight different membrane proteins (Kiebler et al. 1990; Baker et al. 1990; Söllner et al. 1989, 1992; Moczko et al. 1992; Kassenbrock et al. 1993; reviewed by Pfanner et al. 1991). Translocation is initiated by two receptor proteins which bind different subsets of precursor proteins (reviewed by Pfanner and Neupert 1990; Pfanner et al. 1991). Subsequently the preproteins are inserted into a general

insertion pore (GIP) (Pfaller et al. 1988). This step appears to be mediated by the interaction of the positively charged presequences of the preprotein with a cluster of negatively charged amino acids of the outer membrane protein MOM22 (Kiebler et al. 1993). The mechanism of translocation from the outer membrane import machinery to the inner membrane is still unknown. In uncoupled mitochondria, imported proteins can accumulate in the intermembrane space (Rassow and Pfanner 1991). Apparently, the translocation across the outer membrane is inde-

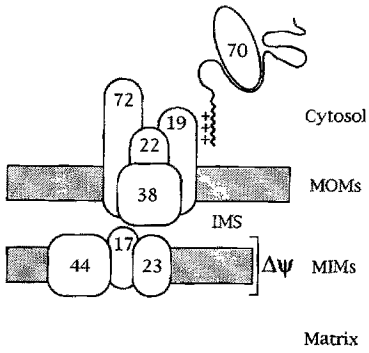

Fig. 5a. Precursor proteins are kept translocation-competent by association with cytosolic hsp70, J components and possibly other chaperone proteins

Fig. 5b. Preproteins bind to import receptors MOM19 and MOM72

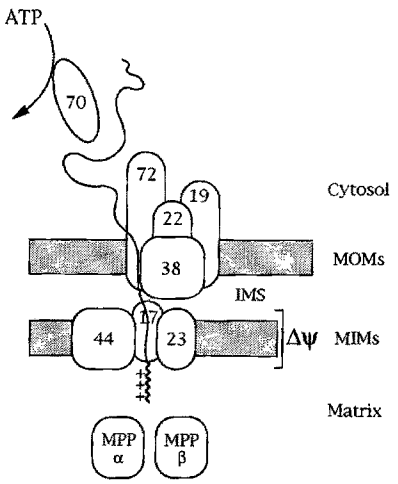

Fig. 5c. Release of hsp70 is ATP-dependent

Fig. 5d. Translocation of the presequence across the inner membrane is driven by the membrane potential. Cleavage of the presequence by MPPα/β can occur during or after translocation

Fig. 5a–i. Model of the function of the protein import apparatus of mitochondria. Mitochondrial outer membrane proteins (*MOMs*) and mitochondrial inner membrane proteins (*MIMs*) are named according to their molecular weights. MOM38 is a major component of the general insertion pore (GIP) which also comprises MOM30, MOM8 and MOM7 (not shown). MOM38 is also known as ISP45 protein, MOM72 is identical to Mas70p. MIM44 is identical to ISP45, and MIM23 is identical to MAS6. *MPP* is the matrix-localized processing peptidase with subunits α and β. *IMS* is the intermembrane space. The model is based on the characterization of mitochondria from fungi *S. cerevisiae* and *N. crassa* . Both mitochondrial membranes are separated by the intermembrane space, IMS. Translocation across the inner membrane is driven by the membrane potential $\Delta\Psi$

Fig. 5e. The peptide in transit adopts an extended structure
Fig. 5f. Mitochondrial hsp70 binds to the unfolded preprotein segment

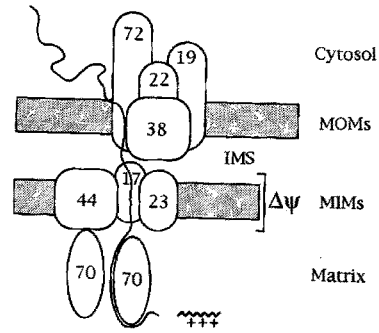

Fig. 5g. Further translocation is driven by mitochondrial hsp70 and needs ATP
Fig. 5h. To some degree, unfolding of the preprotein in transit is a consequence of translocation, and hence an effect of hsp70

Fig. 5i. Folding of the imported protein may involve hsp60

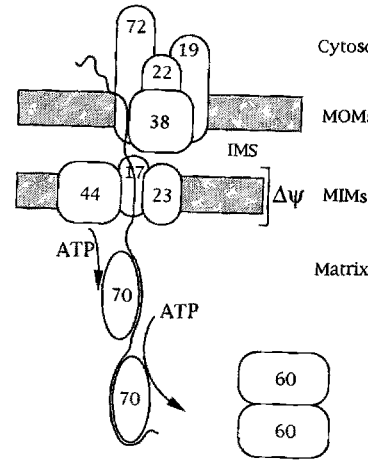

Fig. 5 (*Contd.*)

pendent of the membrane potential across the inner membrane and possibly only driven by the interaction of the presequence with charged amino acids of outer membrane proteins. Only recently the first three components of the inner membrane import machinery, MIM17, MIM23, and MIM44, have been identified (Maarse et al. 1992; Scherer et al. 1992; Horst et al. 1993; Dekker et al. 1993; Emtage and Jensen 1993; Blom et al. 1993). Translocation of proteins across the inner membrane is absolutely dependent on the membrane potential $\Delta\psi$. It is assumed that presequences are translocated across the inner membrane by an electrophoretic effect (Pfanner and Neupert 1985; Martin et al. 1991b). Once the presequence has traversed the inner membrane, the rest of the protein can translocate in the absence of a membrane potential (Schleyer and Neupert 1985). This finding raises the question of which mechanism drives preproteins across the membranes in the $\Delta\psi$-independent part of the translocation.

As we have pointed out in the preceding section, precursor proteins arrive in the mitochondrial matrix as unfolded proteins, probably in extended conformation. This situation resembles the extension of nascent chains at ribosomes. Similar to hsp70 molecules binding to the growing polypeptide at the ribosome, mitochondrial hsp70 binds to proteins which are imported into mitochondria, as soon they become accessible in the matrix. This has been demonstrated by chemical crosslinking and by coimmunoprecipitations of translocation intermediates (Scherer et al. 1990; Ostermann et al. 1990; Manning-Krieg et al. 1991). A fraction of the matrix hsp70 seems to be localized at the inner membrane where it associates with MIM44 (Rassow et al. 1994b). Hsp70 and MIM44 dissociate in the presence of ATP. This suggests a dynamic coupling between the membrane-integrated and soluble components of the import machinery.

The mechanistical implications of the binding of hsp70 to preproteins during translocation have been investigated in detail by characterizing the effects of mutated hsp70s in temperature sensitive mutants

Table 2. Effects of mutations in mitochondrial hsp70 in membrane translocation of preproteins

	SSC1 wild-type	*ssc1-2*	*ssc1-3*
Mutation	–	pro419 ser (peptide-binding domain)	gly56 ser (ATPase-domain)
Ssclp activities	Reversible binding to preproteins	Binding to preproteins, but no release	No binding to preproteins
Activities in protein import	Translocation and unfolding of preproteins	Reduced translocation activity, no unfolding	No translocation, no unfolding
	Translocation of the first part of the presequence of precursor proteins is in all strains solely dependent on $\Delta\psi$ and does not need SSC1 activities		
	Further translocation (translocation of the processed protein) requires binding to hsp70 and hydrolysis of ATP	No further translocation; accumulation of translocation intermediates	No further translocation; accumulation of translocation intermediates
	Preproteins can be imported from urea	Unfolding of preproteins by urea allows complete import	No further translocation of preproteins out of urea

In *S. cerevisiae*, hsp70 of the mitochondrial matrix is encoded by the SSC1 gene.

of the yeast *S. cerevisiae* (Table 2). In yeast, mitochondrial hsp70 is encoded by the gene *SSC1* (Craig et al. 1987, 1989). The first mutation investigated was a point mutation which conferred a defect in the putative peptide-binding domain of the Ssc1 protein. The mutation changes the proline residue in position 419 into a serine residue, the mutant allele is called *ssc1-2*. Isolated hsp70 of this mutant shows normal binding to substrate proteins but impaired release. Mutant yeast cells of this type accumulate several mitochondrial precursor proteins in the cytosol. Precursor proteins which were imported in vitro into isolated mitochondria of this mutant were processed, but accumulated in the translocation sites as translocation intermediates spanning both mitochondrial membranes (Kang et al. 1990). This means that the translocation machinery of these mitochondria is sufficient to perform the first steps of protein import, including the complete translocation of the presequences of the tested precursor proteins. Only the subsequent translocation of the mature protein was strongly reduced. This result is in agreement with the concept of membrane potential driven import of charged presequences. The translocation of the entire preprotein, on the other hand, is obviously dependent on functional matrix hsp70. Therefore it was concluded that hsp70 of the mitochondrial matrix drives the $\Delta\Psi$-independent translocation of preproteins across the mitochondrial membranes (Kang et al. 1990; Neupert et al. 1990).

The inhibition of the translocation of DHFR fusion proteins by methotrexate had indicated the importance of an unfolding of precursor proteins during translocation. All attempts to identify an "unfoldase" at the outer mitochondrial surface have failed. Experiments with mutant hsp70s in yeast have now demonstrated that the unfolding of preproteins at the outer mitochondrial membrane can be due to an indirect effect of mitochondrial hsp70 in the matrix. It is possible to substitute the unfolding activity of hsp70 by an artificial unfolding of the precursor (Gambill et al. 1993). Preproteins which were completely unfolded in 8 *M* urea could not only efficiently be imported into wild-type mitochondria but also into mitochondria containing the defect hsp70 (*ssc1-2*). It can be concluded that under physiological conditions the translocation defect in this mutant is related to its inability to unfold parts of the preprotein. Apparently, the unfolding of carboxy-terminal parts of preproteins at the outer surface of the mitochondrial membranes can be achieved by the action of hsp70 in the matrix. We propose that the unfolding of carboxy-terminal parts occurs in several steps, requiring only low energy inputs. The unfolded segments move across the mitochondrial membranes and are trapped in the matrix by binding to hsp70 molecules. A stepwise binding of hsp70 molecules to the

extended polypeptide chains emerging on the matrix side thus facilitates a stepwise unfolding on the cytosolic side.

This effect was further characterized in a second hsp70 mutant of yeast. After the first hsp70 mutant had been named *ssc1-2*, this second mutant received the name *ssc1-3*. In the *ssc1-3* mutant the glycin of position 56 is changed to serine. This mutation affects the ATPase domain of the hsp70. The consequence of the mutation is a very low affinity of the hsp70 for substrate proteins. Import into *ssc1-3* mitochondria is even more affected than in *ssc1-2* mitochondria. Most remarkably, the effect of this mutation on protein import cannot be restored by an unfolding of the precursors (Gambill et al. 1993). The *ssc1-3* mutation affects a translocation activity which is independent of the unfolding activity. Taken together, the effects of both mutations, *ssc1-2* and *ssc1-3*, suggest a dual role for the mitochondrial hsp70. It is involved in the vectorial movement across the membranes (translocase function) and, at the same time, it mediates the unfolding of the preproteins (unfoldase function).

The dependence on mitochondrial hsp70 is not the same for different precursor proteins. A strong dependence was shown for proteins which are targeted to the mitochondrial matrix. In contrast, proteins which are targeted to the mitochondrial intermembrane space seem to be more or less hsp70 independent. Cytochrome b_2-DHFR fusion proteins which are targeted to the intermembrane space are efficiently imported into *ssc1-2* as well as in *ssc1-3* mitochondria. Deletion of the intermembrane space sorting signal of the cytochrome b_2 presequence results in targeting into the matrix and a strong dependence on the translocase function of mitochondrial hsp70 (Voos et al. 1993).

Interestingly, cytochrome b_2-DHFR fusion proteins with longer b_2 parts which contain a heme-binding domain, need the unfolding activity of hsp70 when imported from reticulocyte lysate in the presence of heme (Voos et al. 1993). The heme stabilizes the tertiary structure of the heme-binding domain of the precursor. In wild-type mitochondria, the unfolding activity of the hsp70 is strong enough to overcome the resistance of the domain against unfolding. In *ssc1-2* and in *ssc1-3* mitochondria the unfolding activity of the mutated hsp70 is impaired, and heme can arrest translocation in a similar way as methotrexate does in the case of the DHFR domain. The different effects of the SSC1 mutations in the import of cytochrome b_2-DHFR proteins indicate the involvement of additional components which recognize the intermembrane space sorting signal. Such factors may partially substitute for the hsp70 translocase function. Apparently, they are not sufficient to unfold the heme-binding domain of cytochrome b_2.

4.1.3 ATP and the Energetics of Protein Unfolding and Translocation

In a previous section (Sect. 3.3) we have already dicussed the ATP requirement for translocation at the cytosolic side of the mitochondrial membranes. Since the subsequent translocation of mitochondrial preproteins is mainly driven by the hsp70 of the mitochondrial matrix, the efficiency of translocation should depend on the presence of ATP in the matrix. If ATP is needed inside the mitochondrion only for the activity of hsp70, depletion of ATP should allow all steps of import which do not involve the hsp70 of the matrix. Consequently, preproteins imported into ATP-depleted mitochondria should traverse the mitochondrial outer membrane and presequences should also be able to traverse the mitochondrial inner membrane. This prediction was corroborated by experiments. Upon import into ATP-depleted mitochondria, the β-subunit of the ATP-synthase does accumulate in the intermembrane space and only the presequence traverses the inner membrane (Rassow and Pfanner 1991; Hwang et al. 1991). The import defects in ATP-depleted mitochondria resemble the effect of the *ssc1-3* mutation, which is characterized by a defect in the ATP-binding domain (Voos et al. 1993; Gambill et al. 1993). The requirement for ATP in the matrix and the involvement of hsp70 are correlated (Wachter et al. 1992; Glick et al. 1993; Cyr et al. 1993; Stuart et al. 1994b). There is no experimental evidence that there are other steps for protein translocation in mitochondria which require ATP than those related to the action of hsp70.

It has been reported that a precursor protein can show an increased protease sensitivity when bound to the outer surface of the mitochondrion (Eilers et al. 1988). This effect was interpreted as partly due to a lipid-mediated conformational change (Endo et al. 1989; Eilers et al. 1988) and partly due to the activity of an unidentified membrane-bound unfoldase (Eilers and Schatz 1988). The latter mechanism may be attributed to the conformational change as a consequence of binding to the mitochondrial import receptors at the outer membrane (reviewed in Pfanner and Neupert 1990). An import mechanism which involves a direct insertion into the lipids of the outer membrane seems to be generally accepted in the case of the import of apocytochrome c. A possible role of lipids in unfolding and import of other precursor proteins has not received further attention. Current research has confirmed a set of distinct proteins as intrinsic components of the mitochondrial import machinery. It was shown that the cytosolic domain of the import receptor MOM72 can bind precursor proteins (in a protease sensitive conformation) in the absence of lipids (Schlossmann et al. 1994).

4.2 Chloroplasts

In chloroplasts preproteins are transported across three different membranes (Fig. 6), the two envelope membranes and the thylakoid membrane (Keegstra 1989). It can be assumed that transport reactions in chloroplasts follow similar principles as in mitochondria or the ER. However, an essential role for chaperone proteins in chloroplast protein import has not been firmly established (Theg and Scott 1993). Translocation across the outer and inner envelope membrane is independent of a membrane potential but dependent on ATP. It has been reported that a chloroplast precursor protein can be bound and inserted into the outer envelope membrane after depletion of ATP. After solubilization of these membranes and fractionation in a sucrose density gradient, an 86-kDa polypeptide and hsp70 were recovered in the same fraction as the arrested preprotein (Waegemann and Soll 1991). The mechanistical importance of the hsp70 in this complex has not yet been determined. After translocation into the stroma a preprotein was found to first bind to hsp70 and then to hsp60 (Tsugeki and Nishimura 1993).

Transport into or across the thylakoid membrane follows different mechanisms, depending on the preprotein (Robinson et al. 1993):

1. Plastocyanin crosses the thylakoid membrane in a reaction that requires only ATP.
2. The 33-kDa subunit of the oxygen-evolving complex (prOE33) and the light-harvesting chlorophyll-binding protein (LHCP) of photosystem II strictly require ATP for integration, but a protonmotive

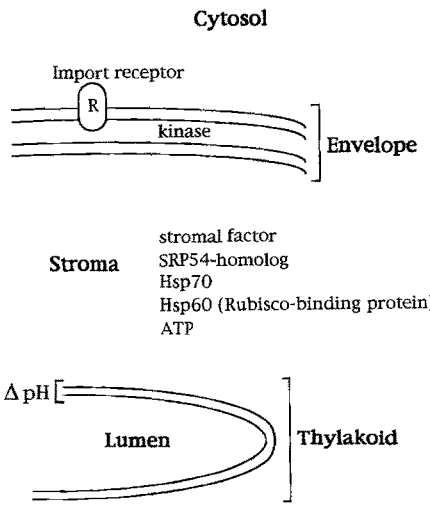

Fig. 6. Import of proteins into chloroplasts. Hydrolysis of ATP is required for binding of preproteins to import receptors. ATP is mainly required in the intermembrane space of the envelope and may be related to protein kinases of the envelope membranes. In the stroma, preproteins bind to stromal hsp70 and other "stromal factors". Rubisco and some other proteins require hsp60 for folding. Translocation across the thylakoid membrane requires ATP and/or ΔpH (See text for references)

force assists in the process (Mould et al. 1991). In chloroplasts it is not the membrane potential Δ which is involved in protein translocation but the chemical gradient ΔpH (Klösgen et al. 1992).

3. The proteins prOE23 and prOE17 are driven across the thylakoid membrane by the proton motive force only, with no apparent requirement for ATP hydrolysis (Mould et al. 1991; Mould and Robinson 1991; Cline et al. 1992).

Some preproteins seem to require a soluble factor from the stroma in order to be transported into the thylakoid lumen (Mould et al. 1991), while others do not (Cline et al. 1992; Bauerle et al. 1991; Mould et al. 1991). Apparently, a stromal hsp70 is one of the "stromal factors" which play a role in these reactions (Yalovsky et al. 1992) but there are indications for additional components (Yuan et al. 1993). One of the stromal factors which target chloroplast proteins to the thylakoid membranes may be the homolog of the 54-kDa subunit of the SRP which was recently identified in *Arabidopsis thaliana* (Franklin and Hoffman 1993). Interesting relationships between protein transport systems of different organisms are indicated by the finding of an SRP system in *E. coli* (recently reviewed by Dobberstein 1994) and of a subunit of a bacterial preprotein translocase (SecY) in a plastid genome (Flachmann et al. 1993).

4.3 Endoplasmic Reticulum

In *S. cerevisiae*, mutations in several genes affect translocation across the ER membrane. One of these is the KAR2 gene which encodes BiP, the hsp70 of the ER lumen (Normington et al. 1989; Rose et al. 1989; Tokunaga et al. 1992). BiP was first identified in mammalian cells as a result of its binding to proteins that accumulate within the lumen of the ER (Haas and Wabl 1983). Similar to the function of mitochondrial hsp70, BiP is an intrinsic component of the protein translocation machinery of the ER. Loss of BiP function blocks translocation (Vogel et al. 1990; Nguyen et al. 1991). As in yeast major components of the ER protein translocation apparatus are known, it was possible to determine the BiP-dependent steps in translocation (Sanders et al. 1992). Preproteins of the secretory pathway first interact with a complex of four different proteins in the ER membrane: Sec62p, Sec63p, a 31.5-kDa glycoprotein and a 23-kDa polypeptide (Novick et al. 1980; Deshaies et al. 1991). In a second step, the preprotein is transferred to Sec61p. This step requires the hydrolysis of ATP. Subsequently the preprotein traverses the ER

membrane and is released from Sec61p. In this sequence of events, BiP is involved in the transfer from the Sec62p-Sec63p complex to Sec61p and also in the translocation across the membrane. This was demonstrated by the analysis of three different mutants of BiP, one of which prohibited the initial interaction with Sec61p, the other two supported an interactwith Sec61p but failed to support translocation. The efficient crosslinking of BiP to preproteins arrested in the ER membrane is consistent with a direct role for BiP in the process of translocation. It is assumed that BiP plays this direct role in facilitating the movement of secretory proteins across the ER membrane, making an initial contact as the secretory protein traverses the membrane, and releasing the polypeptide shortly after its complete translocation into the ER lumen (Sanders et al. 1992). With respect to the initial interaction with Sec62p-Sec63p, it is interesting that Sec63p is an integral membrane protein with a DnaJ-homologous domain facing the ER lumen (Rothblatt et al. 1989; Feldheim et al. 1992). DnaJ is known to interact with DnaK (hsp70) in *E. coli*. Certain alleles of the KAR2 gene, which encodes BiP in yeast, display synthetic lethality with *sec63-1*. In reconstituted proteoliposomes BiP binds to Sec63p and is required for protein translocation (Brodsky et al. 1993; Brodsky and Schekman 1993).

This concept of the function of BiP in the ER is reminiscent of the proposed function of hsp70 in mitochondria. Similar to its function in mitochondria, hsp70 seems to mediate the vectorial movement of preproteins across the ER membrane. As neither Sec61p, Sec62p, nor Sec63p possess a consensus ATP-binding domain, the ATP-requirement of binding to Sec61p and translocation may be attributed to BiP. The interaction of BiP with substrate polypeptides has been demonstrated to be ATP-dependent (Lewis and Pelham 1985; Kassenbrock and Kelly 1989; Flynn et al. 1989). In contrast to mitochondrial hsp70, an indirect unfolding activity (unfolding of protein domains at the trans side of the membrane) has not been found with BiP. This could be due to technical restrictions. With mitochondria the corresponding experiments were performed using urea-denatured precursor proteins. Unfortunately, urea-denatured preproteins are imported into yeast microsomes only with very low efficiency. It should be noted that translocation across the ER membrane does not involve a membrane potential. This may cause some basic differences in the mechanism of translocation across the membranes of the ER and mitochondria. However, in both systems, transport across the yeast ER membrane and transport across the mitochondrial inner membrane, hsp70 seems to be the component which actually drives the translocation of the preproteins.

Table 3. Pro and contra an essential function of hsp70 in the translocation of preproteins across the endoplasmic reticulum membrane in *S. cerevisiae* and in ammals

Method	Reference
S. cerevisiae	
Pro	
Depletion of BiP in vivo by reduced expression of KAR2	Vogel et al. (1990); Nguyen et al. (1991)
Reconstitution of wild-type BiP into mutant membranes	Sanders et al. (1992); Brodsky et al. (1993)
Sec63p, a component of the ER translocation machinery, interacts with BiP	Rothblatt et al. (1989); Deshaies et al. (1991); Feldheim et al. (1992); Brodsky and Schekman (1993)
Contra	–
Mammalian cells	
Pro	
Alkaline extraction at pH 10	Nicchitta and Blobel (1993)
Contra	
In vivo depletion of BiP by antisense gene	Dorner et al. (1988)
Alkaline extraction at pH9	Paver et al. (1989); Bulleid and Freedman (1988)
Reconstitution from detergent solubilized microsomes	Yu et al. (1989); Nicchitta and Blobel (1990); Zimmerman and Walter (1990)
Reconstitution of translocation from purified components	Görlich and Rapoport (1993)

The role of lumenal proteins in protein translocation was also tested with mammalian microsomes. Many data which were obtained with this system are in conflict with the results obtained with yeast. It is not clear whether hsp70 is an essential component of the protein translocation machinery in the mammalian ER (Table 3). The protein concentration of the ER lumen, primarily reflecting the contribution of three proteins, hsp90, PDI and BiP, can approach 100 mg/ml (Koch 1987). In mammalian cells, BiP comprises 5%–10% of the lumenal proteins. There are several reports on effects of the depletion of these lumenal proteins. Most of these reports suggest that lumenal proteins may not be essential for protein translocation. The methods applied were alkaline extraction at pH 9 (Bulleid and Freedmann 1988), reconstitution from partially detergent-solubilized pancreas microsomes (Yu et al. 1989; Zimmermann and Walter 1990), in vivo depletion of BiP by introduction of an antisense gene (Dorner et al. 1988) and reconstitution of a translocation-competent system from purified components (Görlich and Rapoport 1993). To explain the different dependence of

protein translocation on BiP in yeast and in mammalian microsomes, it was suggested that this difference might reflect the difference between post-translational import in yeast versus cotranslational import in the mammalian ER (Görlich and Rapoport 1993). Possibly in the later case translocation is mainly driven by the protein synthesis machinery of the ribosome.

However, in contrast to these reports, other data suggest that in the mammalian ER the mechanism of protein translocation may follow a similar principle as in the ER of yeast. Nicchitta and Blobel (1993) found that canine pancreas microsomes, which have been extensively depleted from lumenal proteins at pH 9.5–10, can partially import preproteins but fail to complete the translocation. They argue that previous investigations may have not completely removed the soluble content of the ER and therefore still retained a considerable translocation activity. They propose a first step of translocation which does not require the activity of lumenal proteins. The free energy change occurring upon interaction of the signal sequence with components of the ER membrane seems to be sufficient to initiate this step, allowing cleavage of the signal peptide. Subsequent steps of translocation are reticuloplasm dependent. There are indications that lumenal hsps are necessary to prevent the release of preproteins into the cytosol after they have inserted into the ER membrane and to bias transit of the nascent chain into the vesicle lumen (Nicchitta and Blobel 1993). In this model, the only difference in the proposed mechanisms in yeast and in mammalian microsomes is the lack of an apparent involvement of BiP in the transfer of preproteins to Sec61p in the mammalian system.

The ATP which is hydrolyzed by BiP inside the ER must be imported from the cytosol. A translocator protein for ATP has not been identified, but it is postulated since import of ATP into mammalian microsomes is saturable and several protein-modifying reagents block its transport (Clairmont et al. 1992).

5 Folding and Sorting of Proteins Inside Organelles

After translocation across biological membranes, proteins undergo a series of chaperone-mediated folding reactions before they become functional as monomers or as subunits of protein complexes. We will briefly review the literature on protein folding in the ER, in mitochondria, and in chloroplasts.

5.1 Protein Folding and Assembly in the ER

While the ER membrane plays a central role in lipid metabolism and biotransformation, the primary function of the ER lumen appears to be the folding, assembly and post-translational modification of proteins. It has been suggested that in the case of the lumen of the ER, protein folding comes close to being its raison d'être (Rowling and Freedman 1993).

BiP is not only involved in the process of translocation of preproteins across the ER membrane but is also an important component of the ER protein-folding machinery. BiP was originally found to bind heavy chains of immunoglobulins in myeloma cell lines that did not produce light chains (Haas and Wabl 1983; Munro and Pelham 1986). However, BiP also binds to heavy chains in cells that make both heavy and light chains and that secrete immunoglobulins. It binds to different intermediates in the assembly process but not to fully assembled immunoglobulins. Besides BiP, hsp90 also associates with unassembled immunoglobulin chains (Melnick et al. 1992) and is thought to function as a chaperone protein (Wiech et al. 1992, 1993). After translocation of the heavy chain, BiP binds mainly to the CH_1 domain, which is the part of the protein to be linked to the light chain (Bole et al. 1986; Hendershot et al. 1987, 1988; Hendershot 1990). A similar result was reported for the trimeric HA (hemagglutinin) protein of the virus surface protein (Hurtley et al. 1989; Segal et al. 1992). BiP binds to those parts of the HA protein where in the HA trimer the three molecules are in contact. Several preproteins were tested for binding to BiP in an in vitro translation/translocation system (Kassenbrock et al. 1988). By immunoprecipitations it was found that reduced or incorrectly disulfide-bonded full-length forms of prolactin associated with BiP. The native disulfide-bonded protein did not bind to BiP. The same was found for the vesicular stomatitis virus G (VSG) protein (Machamer et al. 1990; De Silva et al. 1990). Unglycosylated forms of invertase were found associated with BiP, but not the mature glycosylated form. The exact function of BiP in the assembly of proteins in the ER is not known. However, during folding and assembly, BiP appears to prevent proteins from aggregation. Aggregated proteins stay bound to BiP and are eventually degraded. An interesting example is the common variant of the cystic fibrosis transmembrane conductance regulator (ΔF508 in CTFR). The mutated protein was found associated with BiP and degraded in a pre-Golgi nonlysosomal compartment (Yang et al. 1993). There is a growing number of human diseases which show degradation of mutated

proteins in the ER (Amara et al. 1992). In the case of hemagglutinin, even under normal physiological conditions up to 10% of the protein misfold, are retained in the ER, and are subsequently degraded. Correct folding is the major criterion used by the system that selectively permits exit of secretory and cell-surface proteins from the ER (reviewed by Pelham 1989; Fra and Sitia 1993). The accumulation of mutant or malfolded proteins in the ER causes increased expression of the BiP gene (Kozutsumi et al. 1988). In yeast, a transmembrane protein (ern1p) was identified in the ER which is involved in the transmission of a signal from the ER to the nucleus (Mori et al. 1993).

The mechanism of retention of incompletely folded glycoproteins in the mammalian ER involves calnexin, a transmembrane protein of 88 kDa (Ou et al. 1993; David et al. 1993; Jackson et al. 1994; Rajagopalan et al. 1994; Bergeron et al. 1994). Calnexin is a calcium-binding phosphoprotein. It was suggested that calnexin may be an important component of the quality control system in the ER. As it does not bind the nonglycosylated protein albumin, it is thought to be specific for glycoproteins. Interestingly, calnexin is capable of associating with nascent integral membrane proteins within the membrane: The interaction of calnexin with the class I MHC molecule involve the transmembrane segment of the nascent polypeptide (Margolese et al. 1993). This part of the protein is unavailable to BiP. Calnexin may constitute a unique chaperoning system in the ER membrane which is different not only from the BiP system but from all other soluble chaperone protein systems.

In the lumen of the ER protein folding starts during translocation across the ER membrane. It was found that disulfide bonds in the amino-terminal domain of immunoglobulin light chains form as soon as the domain has traversed the membrane (Bergman and Kuehl 1979a,b). Interestingly, not only intramolecular disulfide bonds are formed during translocation but also disulfide bridges between different chains of the immunoglobulins. Heavy chains are connected to light chains by disulfide bonds while parts of the molecule are still attached to the ribosome at the outer ER surface. Disulfide formation is catalyzed by PDI (reviewed by Noiva and Lennarz 1992). In dog pancreas microsomes, it constitutes 14% of the total lumenal protein. Immunoglobulins can be crosslinked to PDI in vivo (Roth and Pierce 1987). The method of depleting microsomes from lumenal proteins by washing at pH 9 was used to analyze the function of PDI. It was shown that microsomes that had been depleted of lumenal content were defective in cotranslational disulfide formation. The defect could be restored by the incorporation

of purified PDI (Bulleid and Freedman 1988, 1990; Paver et al. 1989). These experiments provided a direct indication of a requirement for PDI in the process of native disulfide formation.

The importance of PDI as a protein-folding catalyst could be demonstrated in vitro. One of the best-characterized folding pathways in vitro is that of bovine pancreatic inhibitor (BPTI), a small 59-residue protein with one domain that contains three disulfide bonds. It was shown that the folding pathway involves rearrangement of non-native disulfide bonds (Creighton 1978) although the predominant intermediates already contain the correct disulfide bonds of the native protein (Weissman and Kim 1991; reviewed by Goldenberg 1992). The refolding of BPTI is catalyzed by PDI (Creighton et al. 1980). This effect was compared with the activity of a preparation of total ER lumenal proteins. The results reveal that pure PDI-catalyzed refolding of BPTI to the same degree as the entire lumenal protein preparation (Zapun et al. 1992).

In other cases PDI is apparently not sufficient to ensure proper folding. PPIs seem to be involved in protein-folding reactions in the ER as well (Lang and Schmid 1988; Bose and Freedman 1992). Cyclosporin A, an inhibitor of cyclophilin, does not inhibit but slows the folding of the monomeric protein transferrin in the ER (Lodish and Kong 1991). Folding of transferrin is not blocked by FK506. Cyclosporin A also slows collagen triple-helix formation in vivo (Steinmann et al. 1991). This was acompanied by a remarkable post-translational overmodification and increased degradation. The effect of cyclosporin A probably is due to an inhibition of a cyclophilin in the ER, albeit indirect effects of the drug cannot be excluded. However, cyclophilin can increase the rate of collagen refolding (Bächinger 1987). A function of a cyclophilin in intracellular protein traffic was shown by the analysis of a drosophila mutant with a deletion of the gene N (Colley et al. 1991; Stamnes et al. 1991; Ondek et al. 1992). Deletion of NinaA causes an inhibition of opsin transport from the ER, leading to an extensive accumulation of ER cisternae in the photoreceptor cells. A function of a FKBP in the ER is suggested by data on a deficiency in amino acid import induced by FK506 in yeast cells (Heitman et al. 1993).

Besides immunoglobulins, hemagglutinin of the influenza virus is probably the best characterized protein with respect to its assembly pathway. It trimerizes in the ER and only the trimer is transported to the Golgi apparatus. This again demonstrates the importance of the ER as a protein-folding compartment. Obviously, the protein-folding machinery of the ER is sufficient to mediate complete folding of the hemagglutinin (Gething et al. 1986; Copeland et al. 1986, 1988; Braakman

et al. 1992a,b; a short review is available by Helenius et al. 1992). It seems to be a general principle that proteins in the secretory pathway oligomerize in the ER (Hurtley and Helenius 1989).

The ER is not only a protein-folding compartment but also a protein modification compartment. Covalent modifications include processing by the signal peptidase, O- and N-glycosylation, trimming of carbonhydrate groups, carboxylation and hydroxylation of amino acid residues. It will be interesting to investigate the cooperation of the involved enzymes with the components of the ER chaperone system.

5.2 The Sequential Action of Hsp70 and Hsp60 in Mitochondria

In contrast to the ER, in mitochondria the complexity of the protein-folding machinery is not due to a plurality of protein modification enzymes, but to the presence of an additional chaperone system. Besides a hsp70 system, mitochondria also contain a hsp60 system. Newly imported preproteins need the interaction with hsp70 as part of the translocation machinery. Therefore, folding at hsp60 can only take place after release from hsp70 (Kang et al. 1990). A sequential action of both chaperone systems has been demonstrated for the folding pathway of a subunit of the matrix-localized processing enzyme MPP (Manning-Krieg et al. 1991) and medium-chain acyl-CoA dehydrogenase (Saijo et al. 1994). But possibly several different folding pathways are used in mitochondria, some of them restricted to the hsp70 system, others depending on both, the hsp70 system and the hsp60 system.

The function of mitochondrial hsp60 was primarily investigated in *N. crassa* and in *S. cerevisiae*. Hsp70 and hsp60 were also found in mammalian mitochondria and are expected to act similar in all eukaryotes (Mizzen et al. 1989). In yeast, hsp60 was detected by the analysis of temperature sensitive strains which were impaired in mitochondrial import functions. Among these *mif* mutants, *mif4* affected the gene encoding mitochondrial hsp60 (Cheng et al. 1989; McMullin and Hallberg 1988; Reading et al. 1989). Isolated mitochondria of this strain showed normal import but no assembly of several proteins. Later it was shown that folding of these proteins in wild-type mitochondria of *N. crassa* requires complex formation with hsp60 and ATP hydrolysis (Ostermann et al. 1989). Efficient folding of newly imported hsp60 monomers needs functional hsp60 in the mitochondrial matrix (Cheng et al. 1990). The requirement for hsp60 in the import and folding of some mitochondrial proteins is still a matter of controversal discussion

and related to the question of the exact import pathway proteins take in mitochondria. Some data indicate that cytochrome b_2 involves complete or partial import into the mitochondrial matrix and interaction with hsp60 prior to transport to the intermembrane space (Koll et al. 1992). Other data are in conflict with these observations and seem to exclude interaction with hsp60 (Glick et al. 1992; Hallberg et al. 1993). The mechanism of hsp60-mediated protein folding in mitochondria is thought to follow the principles described for the GroEL/ES system of *E. coli* (Mendoza et al. 1991). A co-chaperonin cpn10 of mitochondrial hsp60 has been identified in yeast (Lubben et al. 1990; Rospert et al. 1993a,b) and in mammals (Hartman et al. 1992; Ryan et al. 1994). A human mitochondriopathy caused by a defect in hsp60 function was recently reported (Agsteribbe et al. 1993).

Mitochondria contain at least two PPIs, i.e., a cyclophilin (Tropschug et al. 1988, 1989; McGuinness et al. 1990) and a FKBP (Tropschug et al. 1990). The effects of the deletion of mitochondrcyclophilin were investigated in *N. crassa*. It was found that imported DHFR adopted its native conformation in wild-type mitochondria, but that folding was impaired in the deletion mutant. Non-native DHFR reversibly accumulated at mitochondrial hsp70 and hsp60 (Rassow et al. 1994a). These observations demonstrate a protein-folding activity of cyclophilin in vivo and they indicate that mitochondrial hsp70 and hsp60 play a role in the recovery of misfolded proteins, similar to BiP in the ER.

5.3 Protein Folding in Chloroplasts

The conservation of the mechanism of protein folding by different chaperonins has become evident in work on the chloroplast enzyme ribulose bisphosphate carboxylase (rubisco). The complete rubisco enzyme is a protein complex of 500 kDa, it comprises eight large subunits and eight small subunits. The large subunits are synthesized inside the chloroplast and associate with the chaperonin "rubisco binding protein" prior to their assembly (Barraclough and Ellis 1980; Ellis and Hemmingsen 1989; Roy 1989). Actually, this observation was the basis for the concept of "molecular chaperones" (Ellis 1987). The small subunits of rubisco are synthesized in the cytosol and are subsequently imported into the chloroplast. Inside the organelle they bind to the same chaperonin as the large subunits (Gatenby et al. 1988; Gatenby and Ellis 1990). Rubisco also can be folded by *E. coli*-GroEL or by mitochondrial hsp60 from yeast (Hemmingsen et al. 1988). All three chaperonins can efficiently

cooperate with the *E. coli* co-chaperonin GroES (Goloubinoff et al. 1989a,b). The chloroplast chaperonin was named after its rubisco-binding activity, but obviously it is involved in the folding of several other proteins which are imported into chloroplasts (Lubben et al. 1989).

6 Perspectives

Chaperone proteins are involved in protein translocation across many different membranes and serve different cellular functions. These are the biogenesis of organelles (chloroplasts, mitochondria, peroxisomes), the export of proteins (via the ER) and gene regulation (by steroid hormone receptors). The probably most important aim of current research is the elucidation of the functional significance of molecular chaperones in these systems. What is the function of hsp90 in the steroid hormone receptor complexes? Does hsp90 play an important role in the folding of proteins in the cell? What are the functions of the different hsp70s in the cytosol? What is the relevance of mitochondrial hsp60 in protein import and assembly? The hsp60 of mitochondria has been one of the first chaperone proteins which were analyzed as mediators of protein folding. However, it is still unclear by which properties its substrate proteins are defined. As mechanistic studies are often restricted to the characterization of isolated components, it will be a major task to determine the interactions of different chaperone proteins and protein-folding enzymes in the cell. The enzymology of protein folding will have to consider the extremely high protein concentration in vivo. These projects will require the measurement of the affinities and specificities of chaperone proteins. Moreover, it is very likely that many eukaryotic molecular chaperones are still to be identified. Despite of enormous progress in the understanding of protein-folding reactions, it is today still impossible to estimate the complexity of the chaperoning systems of eukaryotic cells.

Acknowledgements. We are grateful to many colleagues for their contributions to the work reviewed here and for sharing unpublished information with us. Work of the authors' laboratory was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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Editor-in-charge: Professor H. GRUNICKE

Heteroreceptor-Mediated Modulation of Noradrenaline and Acetylcholine Release from Peripheral Nerves

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Contents

1 Introduction 266

2 Heteroreceptors Modulating Noradrenaline Release 267
- 2.1 Muscarinic Receptors 267
- 2.2 Nicotinic Receptors 282
- 2.3 Dopamine Receptors 283
- 2.4 β-Adrenoceptors 285
- 2.5 5-Hydroxytryptamine Receptors 286
- 2.6 Histamine Receptors 290
- 2.7 Imidazoline Receptors 296
- 2.8 Adenosine Receptors/Purinoceptors 297
- 2.9 Prostanoid and Leukotriene Receptors 304
- 2.10 Amino Acid Receptors 305
- 2.11 Nitric Oxide, Endothelin Receptors, and Epithelial Factors 311
- 2.12 Atrial Natriuretic Factor 313
- 2.13 Angiotensin Receptors 314
- 2.14 Neuropeptide Y Receptors 318
- 2.15 Sensory Neurotransmitters 319
- 2.16 Opioid Receptors 324
- 2.17 Other Neuroactive Polypeptides 325

3 Heteroreceptors Modulating Acetylcholine Release 327
- 3.1 Localization of Prejunctional Receptors 327
- 3.2 Adrenoceptors 329
- 3.3 Dopamine Receptors 341
- 3.4 5-Hydroxytryptamine Receptors 341
- 3.5 Histamine Receptors 349
- 3.6 Adenosine Receptors/Purinoceptors 350
- 3.7 Prostanoid and Leukotriene Receptors 351
- 3.8 Amino Acid Receptors 360
- 3.9 Nitric Oxide and Vasoactive Intestinal Peptide Receptors 364
- 3.10 Epithelium-Derived Inhibitory Factor 368
- 3.11 Tachykinin and Calcitonin Gene-Related Peptide Receptors 369

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3.12 Opioid Receptors 373
3.13 Somatostatin Receptors 373
3.14 Other Neuroactive Peptide Receptors 375
4 Concluding Remarks 377
References 380

1 Introduction

Currently, prejunctional receptors modulating transmitter release from postganglionic neurones are divided into hetero- and autoreceptors (Starke et al. 1989). The term "prejunctional", often used synonymously with "presynaptic", receptor is preferred in the present review as the neuroeffector junction at peripheral noradrenergic and cholinergic nerve terminals is not a true synapse, and lateral diffusion of transmitter over distances of at least 0.1–10 μm is the rule, judged from the "neuromuscular interval" in the adrenergically innervated smooth muscle (Trendelenburg 1972). Both the terms "prejunctional" and "heteroreceptor" became common usage only several years after the underlying phenomenon had been defined as a physiological mechanism present in the heart and described under a different designation: the muscarinic inhibition of noradrenaline release from postganglionic sympathetic nerve fibres (Lindmar et al. 1968; Löffelholz and Muscholl 1969; Muscholl 1970). The acetylcholine receptor had been characterized by a series of muscarinic agonists and differentiated from a possible nicotinic site, and its effector ascertained by measurement of noradrenaline release evoked by postganglionic electrical nerve stimulation or stimulation of postganglionic nicotinic receptors, as opposed to tyramine-induced noradrenaline release that was not modulated. Activation of the release-inhibiting receptor by vagus nerve stimulation showed that the mechanism was not merely a pharmacological oddity but a physiologically relevant factor (Löffelholz and Muscholl 1970). For reasons discussed in Sect. 3.1, the counterpart of the muscarinic inhibition of noradrenaline release, the α-adrenoceptor-mediated inhibition of acetylcholine release (Paton and Vizi 1969) could not with certainty be allocated to receptors situated either at a ganglionic or at a postganglionic terminal neuronal site, or both.

As reviewed in detail by Starke et al. (1989) the direct evidence for *prejunctional autoreceptors* dates from several papers published simultaneously in 1971, and both the hetero- and the autoreceptor-mediated control of neurotransmitter release have been focal points of neuro-

biological research for the past 26 years. In the first review papers on *heteroreceptor*-controlled transmitter release, only a dozen original sources were quoted, and all were concerned exclusively with muscarinic inhibition of noradrenaline release (Kosterlitz and Lees 1972; Muscholl 1973). Nowadays, compelling evidence for dozens of heteroreceptors including subtypes acting on numerous transmitter systems has accumulated. For the present review, we confine ourselves to the most amply investigated neurotransmitters in the periphery, noradrenaline and acetylcholine (with the exception of presynaptic receptors in the motor endplate which were treated comprehensively by Bowman et al. 1990 and Wessler 1992). Concerning most of the literature from the 1970s, the reader is referred to reviews published around 1980 that will be quoted in the respective sections. Forty-nine percent of the present references originate from 1980 to 1989 and 45% of them from 1990 to 94.

The main emphasis will be given to discussion of those papers in which transmitter release has been determined, but work providing indirect evidence by quantifying end organ responses will not be neglected. In many instances, such observations were the first to throw light on previously unknown prejunctional mechanisms and initiated subsequent work with transmitter overflow determination, which is, as a rule, technically more difficult to perform.

For simplicity no distinction will be made between *basal overflow* and *efflux* of transmitter, or *evoked overflow* and *release*, although there may be reasons to restrict these terms to specifically defined mechanisms. The main emphasis is laid on the existing literature dealing with the action on prejunctional receptors of autacoids, rather than synthetically obtained compounds activating receptors. However, for the purpose of receptor classification into subtypes, pharmacological tools such as selective agonists, and particularly antagonists, are indispensable and will thus be considered. Current receptor nomenclature has been adopted from the recommendations of the IUPHAR committee (TIPS 1993).

2 Heteroreceptors Modulating Noradrenaline Release

2.1 Muscarinic Receptors

The presence of prejunctional muscarinic heteroreceptors on sympathetic nerves is well established (see previous reviews, e.g. Starke 1977;

Table 1. Modulation of sympathetic neurotransmitter overflow/neurotransmission by muscarinic receptors

Species	Tissue	Stimulus + parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Rat	Iris	FS, 3 Hz, 2 min	Methacholine	Inhibition	Pirenzepine, himbacine, methoctramine,	M2	Uptake 1 and uptake 2 inhibited, α_2-adrenoceptors blocked, nearly	Fuder et al. 1989
		^{3}H-NA			4-DAMP, (*R*)- and (*S*)-hexahydrodifenidol-p-F-hexahydrosiladifenidol, UH-AH-37, clozapine, valethamate		total inhibition of evoked overflow; tone by endogenous ACh small and revealed by M_3 selective antagonists only	Bognar et al. 1992
Rat	Trachea	FS trains at 3/15 Hz	Oxotremorine	Inhibition	Scopolamine	M	Uptake 1 and α_2-adrenoceptors blocked; no inhibitory tone by endogenous ACh; muscarinic inhibition independent of epithelium or presence of indomethacin. Endogenous tone by COP; additional indirect not M_2 mediated muscarinic inhibition (perhaps via COP)	Racké et al. 1991
		NA						
		3 Hz			Pirenzepine, methoctramine, p-F-hexahydrosiladifenidol	M_2		Racké et al. 1993
		NA						
Rat	Atria	FS, 2 Hz, 16 or 60 p. ^{3}H	ACh	Inhibition	*N*-Methylatropine	M	Inhibition by muscarinic agonist enhanced during α_2-adrenoceptor blockade: receptor interaction on a prejunctional level	Loiacono et al. 1985

Rat	Heart	SNS, 0.1 Hz, 10 p. ^{3}H-NA	Methacholine, pilocarpine	Inhibition		M	Inhibitors of uptake 1 and 2 and β-adrenoceptors present under conditions of no autoinhibition; irreversible occlusion of prejunctional muscarinic receptors by phenoxybenzamine; large receptor reserve for methacholine, but not for pilocarpine	Fuchs and Fuder 1985
Rat	Heart	SNS, 10 Hz, 10 p. ^{3}H-NA	(+)-, (±)- and (−)-Methacholine	Inhibition		M	Inhibitors of uptake 1 and 2 and adrenoceptors present; irreversible occlusion of prejunctional muscarinic receptors by phenoxybenzamine or propylbenzilyl mustard; stereoselectivity ratio for enantiomers (+)/(−) 650; high receptor reserve for (+)-, less for (−)-methacholine	Fuder and Jung 1985
Rat	Heart	FS, 3 Hz, 20 s ^{3}H or ^{3}H-NA	ACh	Inhibition	Atropine	M	Ouabain induced atropine sensitive inhibition of evoked tritium overflow perhaps via increase in ACh release	Elfellah and Khan 1986
Rat	Heart	SNS, 10 Hz, 10 p. ^{3}H-NA or 3 Hz,	Methacholine	Inhibition	AF-DX 116, hexahydrosila-difenidol	M_2	Evidence for differences between prejunctional muscarinic receptors on noradrenergic nerves in	Fuder et al. 1985a; Dammann et al. 1989

Table 1 (*Contd.*)

Species	Tissue	Stimulus + parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
		1 min ^3H					the heart (later termed M_2) versus cholinergic nerves (M_3) in guinea-pig ileum	
Rat	Heart	SNS, 2 Hz VNS, 15 Hz NA	Endogenous ACh, methacholine	Inhibition		M	Ischemia reduces muscarinic inhibition induced by VNS (perhaps by reduction of ACh overflow) and by exogenous agonist (perhaps by dysfunction of prejunctional mechanisms)	Du et al. 1990
Rat	Portal-vein in vivo	SNS, 2 Hz, 2 min	Methacholine	Inhibition	Atropine, pirenzepine, AF-DX 116, 4-DAMP	M_2	Freely moving rats; evoked overflow nearly abolished; clear evidence for functional role in vivo; no tone by endogenous agonist	Remie et al. 1989; Remie et al. 1990
Rat	Stomach	SNS, 2.5–5 Hz, 1 min VNS NA	Endogenous ACh, oxotremorine	Inhibition	Atropine, pirenzepine, methoctramine, 4-DAMP	M_2	Overflow inhibited by exogenous agonist and VNS; α_2-adrenoceptors blocked; prejunctional inhibition NEM, but not PTX, sensitive	Yokotani and Osumi; 1993
Rat	Bladder base	FS, 2 Hz, 2 min ^3H	Oxotremorine	Inhibition	Atropine	M	Inhibitory tone by endogenous agonist	Somogyi and de Groat 1990

	and body						(70% increase by atropine)	
Mouse	Atria	FS, 3 Hz, 1 min ^{3}H	McN A-343 carbachol	Facilitation Inhibition	Atropine, pirenzepine, dicyclomine, methoctramine	M_1 M_2	Endogenous tone for inhibitory, not for facilitatory receptors; facilitation mediated by activation of protein kinase C	Costa and Majewski 1991; Costa et al. 1993
Guinea pig	Iris	FS, 3 or 5.5 Hz, 2 min ^{3}H-NA	Methacholine	Inhibition	Methoctramine, himbacine, AF-DX 116, hexahydrosiladifenidol	M_2	Uptake 1 and 2 blocked or not; non-specific effects of methoctramine	Fuder et al. 1989 Bognar et al. 1989 Dammann et al. 1989
Guinea pig	Nasal muscosa	FS, 10 Hz, 30 s ^{3}H	ACh	Inhibition	Atropine	M	Concentration-dependent inhibition by ACh, atropine sensitive	Kubo et al. 1989
Guinea pig	Trachea	SNS, 40 Hz, 5 s, relaxation	ACh, pilocarpine	Inhibition	Atropine, methoctramine, hexahydrosiladifenidol	M_3?	No antagonist affinity determined	Pendry and Maclagan 1991a
Guinea pig	Trachea	SNS, VNS, relaxation	Endogenous ACh	Inhibition	Atropine	M	Muscarinic inhibition after VNS. enhanced by physostigmine	Pendry and Maclagan 1991b
Guinea pig	Trachea	FS, 3 Hz, 540 p. NA	Oxotremorine	Inhibition	Pirenzepine, methoctramine, p-F-hexahydrosiladifenidol	M_2	Uptake 1 and α_2-adrenoceptors blocked; for detailed discussion see text	Racke et al. 1992b
Guinea pig	Atria	FS, 5 Hz, 150 p.	ACh, methacholine	Inhibition	Atropine	M	Inhibitors of uptake 1 and 2 and adrenoceptors present; only small extent	Fuder et al. 1985b

Table 1 (*Contd.*)

Species	Tissue	Stimulus + parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
		^{3}H-NA					of slow desensitization of muscarinic inhibition to ACh	
Guinea pig	Atria	FS, 2 Hz, 2 min	Physostigmine, oxotremorine	Inhibition	Atropine, pancuronium, pipecuronium	M_2	Uptake 1 blocked or not; inhibitory tone by endogenous ACh, increased by physostigmine; atropine equipotent at prejunctional autoreceptors and heteroreceptors, but pancuronium and pipecuronium more potent at heteroreceptors	Földes et al. 1989; Manabe et al. 1991
		^{3}H or ^{3}H-NA						
Guinea pig	Carotid artery	FS, 2 Hz	McN A-343, ACh, carbachol	Facilitation	N-methylatropine, pirenzepine, methoctramine, p-F-hexahydrosiladifenidol	M_1	No facilitatory tone by endogenous agonist	Casado et al. 1992
		^{3}H						
Guinea pig	Ileum	FS, 1.5 Hz, 300 p.	Oxotremorine	Inhibition	Atropine	M	Uptake 1 and 2 blocked; physostigmine and atropine alone inhibited and enhanced, respectively, evoked overflow; inhibitory tone by endogenous agonist increased by physostigmine	Alberts and Stjärne 1982
		^{3}H						

Guinea pig	Sub-mucous plexus	focal FS i.p.s.p.		Inhibition	Pirenzepine, 4-DAMP	not M_1, (M_3?)	High affinity of 4-DAMP (pA_2 8.7) points towards presence of M_3 subtype; effect of low antagonist concentrations on their own compatible with tone by endogenous agonist	North et al. 1985
Rabbit	Iris ciliary body	FS, 10 Hz, 300 p. ^3H	Carbachol	Inhibition	Atropine	M	Uptake 1 inhibited, indomethacin present; small inhibitory tone by endogenous agonist; pilocarpine inactive	Jumblatt and North1988
Rabbit	Iris	FS, 5.5 Hz, 2 min ^3H-NA	Methacholine	Inhibition	Atropine, pirenzepine, himbacine, hexahydrosiladifenidol	M_2	Uptake 1 and 2 and α_2-adrenoceptors not blocked, evoked overflow nearly abolished; pilocarpine acts as a presynaptic agonist at 3 Hz, as antagonist at 10 Hz where inhibitory tone by endogenous ACh can be shown	Bognar et al. 1989
		FS, 3/10 Hz ^3H-NA	Pilocarpine					Bognar et al. 1988
Rabbit	Atria	FS, 2 Hz, 90 s ^3H	Carbachol	Inhibition	Atropine	M	Facilitatory effect of angiotensin II enhanced during muscarinic inhibition: prejunctional receptor interaction postulated	García-Sevilla et al. 1985
Rabbit	Atria (perfused)	SNS, 3 Hz, VNS, 1–20 Hz, ^3H-NA ^{14}C-ACh	Endogenous ACh, methacholine	Inhibition	Atropine, N-methylatropine	M	VNS inhibits evoked overflow; inhibition not increased by physostigmine; inhibition correlated with ^{14}C-ACh overflow	Muscholl and Muth 1982

Table 1 (*Contd.*)

Species	Tissue	Stimulus + parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
		SNS, 3 Hz, VNS, 3 Hz, ^{3}H-NA ^{14}C-ACh	Endogenous ACh	Inhibition	Atropine	M	Prejunctional interaction dependent on vago-sympathetic impulse interval (inhibition at 3–10 and 200–283 ms, not at 30 and 167 ms)	Habermeier-Muth and Muscholl 1988
Rabbit	Atria	SNS, VNS, 3 Hz, 3 min each NA ^{14}C-ACh	Endogenous ACh	Inhibition facilitation	Pirenzepine, AF-DX 116	M_2, M_1	VNS inhibited evoked NA overflow via AF-DX 116 sensitive M_2 receptors at all vago-sympathetic impulse intervals (3–233 ms), but at around 100 ms only in the presence of pirenzepine which prevented an M_1-receptor mediated facilitation occurring around an impulse interval of 100 ms	Habermeier-Muth et al. 1990
Rabbit	Atria	FS, 2 Hz, 2 min ^{3}H	Oxotremorine	Inhibition	Atropine	M	Antimuscarinic effects of amitriptyline, imipramine and desipramine investigated	Somogyi and Perel 1989
Rabbit	Cerebral artery	FS, 8 Hz, 1 min ^{3}H	ACh	Inhibition	Atropine	M	No tone by endogenous agonist	Duckles and Kennedy 1982

Rabbit	Ear artery	FS, 3 Hz, trains of 5 10 s Pressure increase	Carbachol	Inhibition	8, e.g., telenzepine, trihexyphenidyl, AF-DX 116, himbacine, hexahydrosiladifenidol	M? not M_{1-3}	Spectrum of antagonist activity incompatible with M_{1-3} subtypes, classification still unclear; receptor possibly a species (rabbit) variant	Darroch et al. 1992
Rabbit	Pulmonary artery	FS, 2 Hz, 10s, contraction	ACh	Inhibition		M	No evidence for influence of endothelium on muscarinic inhibition	Loiacono and Story 1984
Rabbit	Jejunum	SNS/FS ^{3}H or ^{3}H-NA	ACh	Inhibition	Atropine	M	Inhibitory tone by endogenous agonist upon concomitant vagal and sympathetic nerve stimulation	Manber and Gershon 1979
Rabbit	Vas deferens	FS, 0.05 Hz twitch	McN A-343	Inhibition	11, e.g. pirenzepine, himbacine, AF-DX 116, 4-DAMP, etc.	.M_1	Partly guanethidine sensitive neurogenic twitch mainly due to ATP	Eltze 1988
Rabbit	Vas deferens	FS, 1 Hz, 30s NA	e.g. 4-Cl-McN A-343, methacholine	Inhibition	Atropine, (*R*)-trihexyphenidyl, pirenzepine, himbacine, methoctramine	M_1	Agonist and antagonist potency well correlated with data from inhibition of neurogenic twitch; rabbit homologue of rat M_1 receptor or M_4 receptor not excluded	Grimm et al. 1994
Rabbit	Corpus cavernosum	FS, 2/20 Hz, 3 min ^{3}H	ACh	Inhibition	Atropine	M	Inhibitory tone by endogenous ACh	De Tejada et al. 1989
Rabbit	Urethra	FS, 10 Hz, 1 min; 5 Hz, 1 min ^{3}H	Carbachol	Inhibition	Atropine Scopolamine	M	Moderate inhibitory tone by endogenous agonist	Mattiasson et al. 1984; Mattiasson et al. 1987

Table 1 (*Contd.*)

Species	Tissue	Stimulus + parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Cat	Cerebral artery	FS, 2 Hz, 1 min ^{3}H	ACh McN A-343	Inhibition	Atropine, pirenzepine, AF-DX 116, 4-DAMP	M_2 (M_1?)	No true antagonist affinity constants estimated	Alonso et al. 1991
Cat	Femoral artery	FS, 4 Hz, 1 min ^{3}H	Carbachol	Inhibition	Atropine, pirenzepine, AF-DX 116, 4-DAMP	M_3?	No true antagonist affinity constants determined	Fernándes et al. 1991
Cat	Urethra	FS, 5 Hz, 1 min ^{3}H	Carbachol	Inhibition	Scopolamine	M	Small inhibitory tone by endogenous agonist	Mattiasson et al. 1987
Dog	Nasal mucosa	FS, 10 Hz ^{3}H	ACh	Inhibition	Atropine	M	Concentration dependent inhibition by ACh atropine sensitive; no tone by endogenous agonist	Jackson and Steele 1985
Dog	Airways	FS, 2 Hz ^{3}H-NA	ACh	Inhibition	Atropine	M	Tyramine induced overflow of ^{3}H-NA unaffected by ACh	Russell and Bartlett 1981
Dog	Heart in vivo	Sympathetic tone NA	Vagal reflex	Inhibition	*N*-methylatropine	M	Decrease by vagal reflex activation of catecholamines in coronary sinus blood; decrease in heart rate and ventricular pressure by prejunctional inhibition	Lavallée et al. 1980
Dog	Heart in vivo	SNS, VNS heart rate	Endogenous neurotransmitter	Inhibition		M?	Decrease upon VNS of heart rate increase induced by SNS; prejunctional inhibition by VNS	Revington and McCloskey 1990

Dog	Coronary artery	FS, 2 Hz, 6 min ^{3}H-NA	ACh	Inhibition	Atropine	M	Small inhibitory tone by endogenous agonist	Cohen et al. 1984
Dog	Mesenteric artery	FS, 5 Hz, 3 min ^{3}H	ACh	Inhibition		M	Muscarinic inhibition not mediated by endothelium-derived factors, EDRF did not cause prejunctional inhibition	Toda et al. 1990
Dog	Saphenous vein	FS, 2 Hz, contraction	ACh	Inhibition	Atropine, pirenzepine, gallamine	M_2	Prejunctional M_2 type in an organ with postjunctional M_1 muscarinic receptors	O'Rourke and Vanhoutte 1987
Dog in vivo	Gracilis muscle	SNS 2 Hz, 2 min NA	ACh	Inhibition	Atropine	M	Uptake 1 blocked; muscarinic inhibition independent of moderate changes in blood flow; no prejunctional influence by VIP and substance P	Kahan et al. 1985
Dog	Bladder neck	FS, 5 Hz 3 min ^{3}H	ACh	Inhibition	Atropine	M	Uptake 1 and 2 blocked; small inhibitory tone by endogenous agonist	Mutoh et al. 1987
Dog	Retractor penis	FS, 0.2–20 Hz contraction and e.j.p.	Carbachol	Inhibition	Atropine	M	Physostigmine active without exogenous agonist indicating inhibitory tone upon cholinesterase blockade by endogenous agonist	Kinekawa et al. 1984
Cattle	Cerebral artery	FS, 4 Hz, 1 min ^{3}H	Carbachol	Inhibition	Atropine, pirenzepine, AF-DX 116, methoctramine, 4-DAMP	M_2 and M_3?	No inhibitory tone by endogenous ACh; antagonist affinity not determined	Ferrer et al. 1992

Table 1 (*Contd.*)

Species	Tissue	Stimulus + parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Man	Iris ciliary body	FS, 5 Hz, 300 p. ^{3}H	Muscarine	Inhibition	Atropine	M	Small inhibitory tone by endogenous agonist	Jumblatt et al. 1993
Man	Saphenous vein	FS, 8 Hz, 2 min ^{3}H-NA	ACh	Inhibition	Atropine	M	Muscarinic agonist causes not only prejuctional inhibition but also postjunctional decrease of response to exogenous NA	Rorie et al. 1981
Man	Vas deferens	FS, 5 Hz, 1 min ^{3}H	Carbachol	None	Scopolamine		No agonist or antagonist effect observed, but moduation by α_2-adrenergic agonist and antagonist	Hedlund et al. 1985
Man	Corpus cavern-osum	FS, 2 Hz, 3 min ^{3}H	Physostig-mine	Inhibition	Atropine	M	Only small inhibition probably due to increase by physostigmine in autoinhibition of ACh release	De Tejada et al. 1989
Man	Urethra	FS, 10/5 Hz, 1 min ^{3}H	Carbachol	Inhibition	Scopolamine	M	Small inhibitory tone by endogenous agonist	Mattiasson et al. 1984, 1987

Please note that receptor classification under Subtype may not correspond to that given in the paper quoted, but was adjusted to the current classification according to TIPS nomenclature supplement (TIPS 1993). Antagonists or agonists listed were not necessarily active, but their affinity or potency or lack of action was used for receptor (subtype) classification. For chemical designation of code names see publications quoted.

FS, field stimulation; SNS, sympathetic nerve stimulation; VNS, vagal nerve stimulation; NA, noradrenaline; ACh, acetylcholine; NEM, N-ethylmaleimide; PTX, pertussis toxin; p., pulses; e.j.p., excitatory junctional potential; i.p.s.p., inhibitory postsynaptic potential; COP, cyclooxygenase products

Muscholl 1979, 1980; Vanhoutte et al. 1981; Rand et al. 1990). Morphological and biochemical evidence shows bidirectional axonal transport of muscarinic receptors in peripheral noradrenergic nerves (see Laduron 1985). Chemical sympathectomy in rats by 6-hydroxydopamine decreased the number of muscarinic binding sites in the spleen, a tissue with few postjunctional muscarinic receptors, indicating a prejunctional location of the binding sites lost (Yamada et al. 1982). Only recently, heterogeneities of muscarinic heteroreceptors with respect to functional response (prejunctional facilitation or inhibition) and to receptor subtypes (M_1–M_3) were recognized. Thus, heteroreceptors sensitive to low pirenzepine concentrations which probably correspond to the M_1 subtype may *facilitate* noradrenaline release in rabbit atria (Habermeier-Muth et al. 1990), mouse atria (Costa and Majewski 1991; Costa et al. 1993) and the guinea-pig carotid artery (Casado et al. 1992). On the other hand, heteroreceptors likely to belong to the M_1 subtype *inhibit* the neurogenic twitch response to the release of adenosine triphosphate (ATP) (Eltze 1988) and of endogenous noradrenaline in the rabbit vas deferens (Grimm et al. 1994). The majority of prejunctional heteroreceptors found in virtually every species including man (see Table 1) which inhibit noradrenaline release has been classified as M_2. In a few preparations such as guinea-pig trachea in vivo (Pendry and Maclagan 1991a), cat femoral artery (Fernándes et al. 1991) and bovine cerebral arteries (Ferrer et al. 1992), however, M_3 receptors may contribute, or solely be responsible for a muscarinic inhibition. So far this suggestion has not been fully substantiated by a determination of affinity constants of subtype selective antagonists for these prejunctional heteroreceptors. In the guinea-pig trachea in vitro, the receptor responsible for direct inhibition of noradrenaline release was exclusively of the M_2 type (Racké et al. 1992b) and not, as expected from indirect evidence obtained in in vivo studies, an M_3 type (see above). Interestingly, in the rat trachea, oxotremorine inhibited the noradrenaline release directly via an M_2 receptor and indirectly, involving a cyclooxygenase product generated by an (additional?) muscarinic receptor subtype (Racké et al. 1993). In many tissues, the subtypes of inhibitory muscarinic receptors have not been determined so far. An atypical receptor different from M_1–M_3 subtypes is responsible for muscarinic inhibition in the rabbit ear artery (Darroch et al. 1992).

The inhibitory effect of acetylcholine released upon vagal nerve stimulation or exogenous muscarinic agonists on the release of noradrenaline in the heart is well established (Löffelholz and Muscholl 1970; Muscholl 1980; Levy 1984). The prejunctional vagosympathethic

interaction may, in contrast to its postsynaptic counterpart, have only a limited functional relevance in some sections of the heart or under some experimental conditions. Thus, at the atrioventricular node, the extent of a prejunctional vagosympathetic interaction depends on the experimental setup; some authors found little effect (Levy 1984) while others reported that a prejunctional component was probably involved (Takahashi and Zipes 1983; Salata et al. 1986). Whereas the postjunctional interaction at the sinus node of the dog heart was evident under all conditions, a prejunctional vagal suppression of sympathetic tachycardia was suggested to be of functional relevance only when large amounts of acetylcholine are released (Kimura et al. 1985).

Muscarinic facilitation of noradrenaline release has only recently been detected (Habermeier-Muth et al. 1990; Costa and Majewski 1991; Casado et al. 1992). In the heart, facilitatory and inhibitory muscarinic modulation takes place simultaneously. Acetylcholine that is released upon vagal nerve stimulation predominantly inhibits via M_2 receptors the noradrenaline release evoked by concomitant stimulation of the postganglionic sympathetic nerves in rabbit perfused atria at a variety of defined fixed intervals between vagal and sympathetic pulses (Muscholl and Muth 1982; Habermeier-Muth and Muscholl 1988; Habermeier-Muth et al. 1990). Whereas the M_2 inhibitory mechanism is present at vagosympathetic impulse intervals ranging from 3 to 300 ms, the M_1 facilitatory effect is observed only at an interval of 100 ms and is superimposed on the larger inhibitory action (Habermeier-Muth et al. 1990). Judged on the basis of selective antagonist concentrations that are required for blockade of hetero- and autoreceptors, higher biophase concentrations of released acetylcholine are attained at the hetero- than at the autoreceptor. In mouse atria, noradrenaline release evoked by field stimulation was inhibited or facilitated by M_2 or M_1 receptors when activated by exogenous agonists, but the endogenous agonist liberated by field stimulation appeared to cause inhibition only (Costa and Majewski 1991). The facilitation may be caused by activation of protein kinase C (Costa et al. 1993). In contrast, muscarinic inhibition by M_2 receptors may be mediated by an N-ethylmaleimide-sensitive (Yokotani and Osumi 1993), pertussis toxin-insensitive G protein (Yokotani and Osumi 1993; Costa and Majewski 1990) and do not involve cyclic adenosine monophosphate (cAMP)-dependent mechanisms (Costa and Majewski 1990). Evidence for this was provided by Costa and Majewski (1990), who measured the tritium overflow in rat right atrial strips, and by Yokotani and Osumi (1993) who determined the release of endogenous noradrenaline in rat perfused stomach. One

muscarinic mechanism in rat sympathetic neurones was found to consist in a reduction of high-voltage activated Ca^{2+} currents involving a pertussis toxin-sensitive G protein (Song et al. 1991), while low-voltage activated Ca^{2+} or Na^{+} currents were unaffected. In the rat superior cervical ganglion, sympathetic neurones show a slow M_1 receptor-mediated suppression of the M Ca^{2+} current, and a fast pertussis toxin-sensitive suppression of Ca^{2+} current mediated by the M_4 receptor subtype (Bernheim et al. 1992). The diversity and significance of neuronal calcium channels was recently reviewed (Anwyl 1991). Muscarinic inhibition fails to undergo a rapid and strong desensitization in guinea-pig atria (Fuder et al. 1985b).

Under physiological conditions, sympathetic and parasympathetic nerves are stimulated at discrete patterns of different frequencies, and no *simultaneous* stimulation with the *same* frequency occurs as it does during field stimulation of isolated tissues. It is, therefore, difficult to assess the physiological relevance of a muscarinic modulation by endogenous acetylcholine from experiments in which field stimuli were applied. Hence an increase induced by muscarinic receptor antagonists of noradrenaline release evoked by field stimulation per se (see Table 1) cannot be taken as evidence for a physiological role of prejunctional vagosympathetic interactions. Likewise, the absence of field stimulation-evoked effects on noradrenaline overflow of muscarinic receptor antagonists does not necessarily contradict muscarinic modulation of sympathetic nerve function, as it is obvious that a minimum latency of the presynaptic response has to be taken into account (about 20 ms; Habermeier-Muth and Muscholl 1988). On the other hand, vagal or sympathetic nerves can be separately stimulated only in a few preparations and, even if it can be done, the physiological relevance of artificial stimulation patterns is highly questionable. The best evidence for a (patho)physiological role of prejunctional vagosympathetic interactions comes from experiments with reflex-activated sympathetic and/or parasympathetic tone (Lavallé et al. 1980). It has been suggested that cholinergic influence on sympathetic tone might contribute to cardiac electrophysiological instability and vulnerability (for review, see Verrier and Hohnloser 1987).

The muscarinic inhibition induced by exogenous agonists is generally a powerful mechanism to suppress noradrenaline release. Release induced by long trains of stimuli applied at high frequency (see Table 1) or by high potassium (Muscholl 1980) can be depressed. The potency of muscarinic agonists is only moderately dependent on the stimulation frequency. In the rat heart, methacholine abolished totally

the [^{3}H]-noradrenaline overflow evoked by 10 pulses at a frequency of 0.1 Hz (no autoinhibition by endogenous noradrenaline) or 10 Hz (autoinhibition blocked by antagonists; Fuchs and Fuder 1985; Fuder and Jung 1985). At 10 Hz, the concentration of half-maximal inhibition by methacholine was less than fivefold higher than it was at 0.1 Hz. Under conditions of ischaemia, however, the muscarinic inhibition of noradrenaline release by endogenous and exogenous agonists was reduced, presumably due to both hypoxia-induced reduction in acetylcholine release and failure of the prejunctional muscarinic mechanisms (Du et al. 1990). In streptozotocin-induced diabetic rats (but not in control rats), atropine failed to increase the [^{3}H]-noradrenaline overflow evoked by field stimulation, indicating either that the amounts of acetylcholine released were insufficient to cause muscarinic inhibition, or that prejunctional muscarinic mechanisms are not operative in this pathophysiological state (Gando et al. 1993)

Interestingly, muscarinic inhibition of sympathetic neurotransmission in the rabbit pulmonary artery was found to be independent of the functional integrity of the endothelium and hence not mediated via an endothelium-derived factor (Loiacono and Story 1984). It was similarly concluded from experiments on isolated dog mesenteric arteries that muscarinic inhibition did not involve endothelium-derived relaxing factor (EDRF; Toda et al. 1990). The ouabain-induced inhibition of evoked release of labelled and endogenous noradrenaline was enhanced by physostigmine and reduced by atropine, indicating that muscarinic mechanisms are involved (Panek et al. 1986). An interaction between muscarinic inhibition of noradrenaline release with α-adrenergic autoinhibitory feedback mechanisms in rat atria, as suggested by Loiacono et al. (1985), could not be confirmed by others (Boyle and Pollock 1987).

2.2 Nicotinic Receptors

While nicotinic stimulation of resting sympathetic nerves has been studied extensively and has been well established to enhance basal noradrenaline overflow (see Löffelholz 1979) and overflow of cotransmitters such as ATP (Bültmann et al. 1991; von Kügelgen and Starke 1991a), little is known about the involvement of prejunctional nicotinic receptors in the modulation of evoked noradrenaline (or cotransmitter) release. Previous results suggest that stimulation-evoked release of newly incorporated [^{3}H]-noradrenaline is either facilitated in peripheral blood vessels, or inhibited in pial blood vessels (for review, see Nedergaard

1988). In the rat perfused heart, nicotinic agonists failed to affect the release of tritiated noradrenaline (Fuder et al. 1982; Elfellah and Khan 1986). In the guinea-pig vas deferens, besides an increase in basal overflow, a stereoselective nicotinic facilitation of evoked [^{3}H]-noradrenaline release was observed, which underwent desensitization upon prolonged agonist exposure (Todorov et al. 1991). The rank order of agonist potency (DMPP = (−)-nicotine > (+)-nicotine; cytisine and cotinine being inactive) and the insensitivity to α-bungarotoxin pointed towards a similarity between the prejunctional nicotinic receptors on sympathetic nerves and those on motoneurones, and a difference between these and the ganglionic receptors and the postjunctional receptors on striated muscle. Simultaneous activation of inhibitory prejunctional muscarinic receptors functionally counteracted the nicotinic facilitation (Todorov et al. 1991). In the guinea-pig ileum, nicotinic facilitation of the overflow of endogenous noradrenaline evoked by field stimulation in the presence of desipramine was not subject to desensitization (Plenz 1990). Interestingly, hexamethonium decreased the evoked release and, in the presence of physostigmine and scopolamine, the evoked release was enhanced, indicating that endogenous acetylcholine participates in nicotinic facilitation (Plenz 1990). Taken together, there is only little evidence for a physiological role of nicotinic modulation of evoked sympathetic neurotransmitter release, since acetylcholine probably fails to reach concentrations high enough to stimulate the receptors and, moreover, activates muscarinic receptors at much lower concentrations. Whether nicotinic facilitation or inhibition of exocytotic noradrenaline release in peripheral tissues contributes to pathophysiologically relevant nicotine effects remains open.

2.3 Dopamine Receptors

Abundant evidence demonstrates the presence of inhibitory prejunctional dopamine receptors on peripheral sympathetic nerves and has been reviewed critically by Willems et al. (1985). Morphological and biochemical evidence shows that D_2 receptor binding sites disappear after chemical sympathectomy with 6-hydroxydopamine, thereby proving the prejunctional location of dopamine receptors in the rat mesenteric vascular tree and caudal artery (Ricci and Amenta 1990; Mancini et al. 1991). The dopaminergic inhibition may apply not only to the release of noradrenaline but also to a purinergic cotransmitter (possibly ATP). This is suggested by indirect functional evidence in the rat

kidney, where not only release of radioactive noradrenaline but also the purinergic pressure response to sympathetic nerve stimulation was reduced by the D_2 agonist carmoxirole (Rump et al. 1992). The physiological agonist dopamine stimulates dopamine receptors predominantly in species such as cats, dogs and rabbits and reduces the sympathetic neurotransmitter release. In rats and guinea pigs, prejunctional α_2-adrenoceptor stimulation is the dominant effect of dopamine, and not the activation of dopamine receptors. Dopaminergic inhibition was observed in many different organs such as the heart, blood vessels, kidney, spleen and a few others (but not in the vas deferens of the rat, mouse and guinea pig or human bladder; see Willems et al. 1985). The inhibitory receptor is exclusively of the D_2 type.

Whereas there is no doubt about the pharmacological role of dopaminergic inhibition, little evidence supports the view that endogenous dopamine modulates sympathetic neurotransmission to a relevant extent under physiological conditions (Muscholl 1982). In the rabbit heart in vitro, dopamine increased the basal and evoked release of noradrenaline in the absence of cocaine, and inhibited the evoked overflow only in the presence of cocaine (Fuder and Muscholl 1978). Similarly, indirect sympathomimetic effects of dopamine counteracted a prejunctional dopaminergic inhibition in the rabbit in vivo (Ensinger et al. 1985). Moreover, it was concluded that activation of postjunctional vascular dopamine receptors (D_1) leading to a reduction in blood pressure is the dominant mechanism of a dopamine-induced hypotensive effect, rather than prejunctional inhibition of the noradrenaline release rate (Ensinger et al. 1985). This may be different for other agonists at prejunctional dopamine receptors such as bromocriptine or quinpirol which are devoid of indirect sympathomimetic effects and are weak D_1 receptor agonists (Ensinger et al. 1985; Szabo et al. 1992).

It has been suggested previously that prejunctional dopamine agonists may represent promising antihypertensive drugs (Langer and Shepperson 1982). Under chronic treatment with monoamine oxidase inhibitors, hypotension may be the result of the release of dopamine accumulating in sympathetic nerves, which then could reduce sympathetic neurotransmission via prejunctional dopamine receptors (Langer et al. 1987). The physiological role of dopamine as a prejunctional agonist may best be seen in areas where dopamine itself can be released to an appreciable extent from nerves via stimulation. This may be the case in the dog mesentery, where the release of noradrenaline and dopamine induced by high potassium solution was enhanced by sulpiride in the absence and presence of phentolamine (Soares-da-Silva 1987), but

perhaps not in the rat kidney (Rump et al. 1992). Interestingly, prejunctional dopamine receptors maintain their sensitivity with age as suggested from the indirect parameter, contraction to nerve stimulation in rat tail artery (Buchholz et al. 1992). As with many other prejunctional receptors, the effectiveness of dopamine receptor activation correlates inversely with the calcium ion concentration in the medium, the number of pulses in a stimulation train and the frequency (Friedman et al. 1992).

2.4 β-Adrenoceptors

The physiological or pathophysiological role of prejunctional β-adrenoceptors is unclear. There is evidence for facilitatory effects on sympathetic neurotransmission of exogenous β-adrenoceptor agonists which are sensitive to β-adrenoceptor blockade (comprehensively reviewed by Starke 1977; Dahlöf 1981; Misu and Kubo 1986; Rand et al. 1990; Abrahamsen 1991). The facilitation involves not only noradrenaline release but also the release of the cotransmitter neuropeptide Y (NPY) (Dahlöf et al. 1991). Adrenaline, however, which might be the physiological endogenous agonist and would be expected to stimulate β-adrenoceptors better than noradrenaline, facilitates the evoked noradrenaline overflow in most cases only when prejunctional α_2-adrenoceptors are blocked; otherwise it might also cause inhibition (via α_2-adrenoceptors) or have no effect (see Abrahamsen 1991). It was suggested that adrenaline released from the adrenals under stress may be involved in the genesis of hypertension: adrenaline taken up into the sympathetic nerves and released as a cotransmitter in small amounts (insufficient to stimulate α_2-adrenoceptors) could possibly induce facilitation of noradrenaline release and thus contribute to enhanced blood pressure (see Rand et al. 1990). A facilitation of sympathetic neurotransmission upon incorporation of adrenaline into sympathetic storage granules cannot always be documented and, hence, a decrease of noradrenaline release by β-adrenoceptor antagonists is often not observed (see Abrahamsen 1991). Recently, it was shown that chronic treatment with adrenaline of rats in vivo resulted in an increase in endogenous noradrenaline overflow from the kidney perfused in vitro which was not due to β-adrenoceptor-mediated facilitation but rather to inactivation of the α-adrenoceptor-mediated autoinhibition (Schwartz and Eikenburg 1988). Also upon acute adrenaline pretreatment, inhibitory α_2-autoreceptors predominate by far over facilitatory β-adrenoceptors in the rat kidney (Schwartz and

Eikenburg 1989). In the mesentery of rats chronically pretreated with adrenaline, propranolol failed to reduce the evoked noradrenaline release in the absence and presence of phentolamine, despite the fact that adrenaline represents 50% of the catecholamine in the vesicles (Sadeghi and Eikenburg 1992). The role of facilitatory β-adrenoceptors is further complicated by the inhibitory effects of β-agonists sensitive to β-blockade: Isoprenaline facilitated via β_2-adrenoceptors the evoked noradrenaline release from rat atria (in the presence of phentolamine), but in the presence of a β_2-antagonist, an inhibition was observed which was reversed by atenolol, a β_1-selective antagonist (Mian et al. 1992). It was concluded that activation of postjunctional β_1-adrenoceptors induced the release of mediators other than cyclooxygenase products, which modulated noradrenaline release transsynaptically. Similar inhibitory effects were also observed in the mesentery of normotensive rats (Sadeghi and Eikenburg 1993). It is unclear whether the β-mimetic inhibition is exclusively indirect. Indirect β-adrenoceptor effects have been observed: in the pithed rabbit, a β_2-mediated direct facilitation is accompanied by an indirect β_1-mediated increase in evoked noradrenaline release involving renin activity and angiotensin II (Majewski et al. 1985). Angiotensin II, however, is not involved in the isoprenaline-induced facilitation in mouse atria and the rat tail artery (Rajanayagam et al. 1989). In contrast to β_2-adrenoceptor-mediated effects which are well established in vitro (see Abrahamsen 1991) and in vivo (Remie et al. 1988), β_1-mediated facilitation of noradrenaline release is rarely found in vitro, but more often in vivo (see above; Dahlöf 1981; Yokotani et al. 1992).

2.5 5-Hydroxytryptamine Receptors

Until a few years ago, peripheral neuronal 5-hydroxytryptamine (5-HT) receptors could be easily classified in functional terms into facilitatory and inhibitory receptors modulating noradrenaline release (see Fozard 1984; Humphrey 1984). Recent developments in pharmacological knowledge and structural information from genetic codes have led to an explosive increase of receptor subtypes and quick changes in nomenclature (see Humphrey et al. 1993). The pharmacological characterization of prejunctional 5-HT receptors modulating noradrenaline release could not follow nearly as fast, and the identity of receptors listed in Table 2 does not necessarily reflect the new terminology. Moreover, since the functional studies were carried out at a time when the subtype selectivity of 5-HT agonists or antagonists had not been

Table 2. Modulation of sympathetic neurotransmitter overflow/neurotransmission by 5-hydroxytryptamine receptors

Species	Tissue	Stimulus + parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Rat	Pithed rat in vivo	SNS, pressure and/or heart rate	8-OH-DPAT, RU24969	Inhibition		5-HT_1	No antagonist tested, 5-HT_{1A} subtype discussed, but 5-HT_{1B} subtype not excluded. Ketanserin present to block postjunctional 5-HT_2 receptors	Park et al. 1991
			5-HT, 5-Methoxy-tryptamine		Methiothepin			Göthert et al. 1986a
Rat	Kidney (perfused)	SNS, 2 Hz, 20 s ^3H	5-HT, 5-carboxamido-tryptamine, RU24969, 8-OH-DPAT	Inhibition	Methiothepin, metergolin, methysergide, cyproheptadine, ketanserin, mesulergin, etc.	5-HT_1-like (not 5-HT_{1A-C})	Spectrum of agonist and antagonist activity not compatible with 5-HT_{1A-C} subtypes	Charlton et al. 1986
Rat	Vena cava	FS, 0.66 or 2 Hz ^3H	5-HT, RU24969, 5-methoxy-tryptamine, ipsapirone	Inhibition	Methiothepin, propranolol, ketanserin, spiperon	5-HT_{1B}	Spectrum of agonist and antagonist potency best compatible with 5-HT_{1B} subtype; reciprocal interaction between 5-HT_{1B} receptors and α_2-autoreceptors	Göthert et al. 1986a; Molderings et al. 1987; Göthert et al. 1991; Molderings and Göthert 1990
Guinea pig	Inferior mesenteric artery	Focal stimulation, e.j.p.	5-HT	Inhibition	Methiothepin, methysergide, MDL72222	5-HT_1-like	In addition to the inhibitory response, a facilitatory 5-HT_3 receptor mediated stimulation was observed located probably at somatic parts of neurone	Meehan and Kreulen 1991

Table 2 (*Contd.*)

Species	Tissue	Stimulus + parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Rabbit	Ear artery	FS, 1 Hz, 30 p. ^3H	5-HT	Inhibition	Methiothepin, ketanserin	5-HT_1-like	After pre-exposure to 5-HT methiothepin enhanced the evoked NA overflow (but not upon uptake 1 blockade); 5-HT acts as cotransmitter	Meehan and Story 1989
Cat	Nictitating membrane	Pregang-lionic NS, 0.1–3 Hz	5-HT	Facilitation	Methysergide, ketanserin, pindolol, metoclopramide	5-HT_1	Interpretation compli-cated by postjunctional contraction via 5-HT_2 receptors	Park et al. 1991
Dog	Coronary arteries, saphenous vein	FS, 1–2 Hz, 6 min ^3H-NA	5-HT, platelets	Inhibition	Methiothepin	5-HT_1-like	Exogenous 5-HT and 5-HT released from platelets inhibited tritium release in a methiothepin sensitive manner; other platelet mediators caused additional inhibition	Lorenz and Vanhoutte 1985
Dog	Coronary artery (left circumflex)	FS, 2 Hz ^3H-NA, and β-adrenergic relaxation	5-HT	Inhibition	Methiothepin, metergoline, ketanserin	non-5-HT_2	Inhibition independent of uptake 1 and α-adrenoceptor blockade; muscarinic inhibition not blocked by methiothepin	Cohen 1985
Dog	Tibial artery	FS, 2 Hz ^3H-NA and con-	Pre-exposure to 5-HT (incorpor-ated into and	Inhibition	Methiothepin	5-HT1-like	5-HT acts as inhibitory cotransmitter after exposure to 5-HT; cocaine	Cohen 1987

		traction to FS	released from nerves)				sensitive incorporation into nerves; release of labelled 5-HT after pre-exposure TTX sensitive	
Dog	Saphenous vein	FS, 10 Hz, 2.5 min ^{3}H (^{3}H-NA)	5-HT, 8-OH-DPAT	Inhibition	Methiothepin, methysergide, (−)-pindolol, ketanserin	5-HT_1 not 5-HT_{1A} or $_{1B}$	Pre-exposure to exogenous 5-HT decreased fractional rate of release indicating cotransmission and "autoinhibition"	Paiva et al. 1988
Cattle	Ovarian follicle	FS, 5 Hz, 2 min ^{3}H or contration	5-HT, 5-carbox-amidotryptamine, 8-OH-DPAT	Inhibition	Methiothepin, methysergide, cyanopindolol, MDL72222	5-HT_1-like 5-HT_{1D}?	Relative agonist potency and spectrum of antagonist activity does not allow a definitive classification of subtype involved	Kannisto et al. 1987
Pig	Coronary artery	FS, 0.66 Hz ^{3}H	5-HT and many others	Inhibition	Methiothepin, ketanserin, ICS 205-930, propranolol, etc.	5-HT?	None of the antagonists blocked the 5-HT effects; novel subtype discussed	Molderings et al. 1989; Göthert et al. 1991
Man	Saphenous vein	FS, 0.66 or 2 Hz, 3 min ^{3}H	5-HT, 8-OH-DPAT, TVX Q7821, and many others	Inhibition	Methiothepin, propranolol many others	5-HT_1-like, later defined as 5-HT_{1D}	Inhibition by 5-HT and 8-OH-DPAT insensitive to rauwolscine; no clear sub-classification of receptor; uptake 1 and 2 blocked	Göthert et al. 1986b; Molderings et al. 1990; Göthert et al. 1991

For explanations, see Table 1.
5-HT, 5-hydroxytryptamine, serotonin; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin

completely assessed, in some cases the classification must be considered premature or incomplete. Excitatory 5-HT receptors on sympathetic nerves have been reviewed previously (Fozard 1984) and will not be discussed here. In recent years, much work has been devoted to the inhibitory prejunctional 5-HT receptors (see Table 2). Little is known about a physiological function of these inhibitory receptors and the source of an endogenous agonist. One of three possible sources are 5-HT-ergic nerves. In nerve cell cultures from guinea-pig hearts grown in 5-HT supplemented medium (but not in those grown in 5-HT depleted medium), morphological evidence shows that nerves and some other cells store 5-HT and may be considered as possible sources (Hassall and Burnstock 1987). Another source may be blood platelets which contain and release large amounts of 5-HT that could then be taken up by amine-handling nerves such as postganglionic sympathetic fibres (Fozard and Mwaluko 1973). In the dog coronary artery and saphenous vein, exposure to aggregating platelets or exogenous 5-HT was followed by a decrease in evoked overflow of [^{3}H]-noradrenaline; the inhibition was reversed by methiothepin (Lorenz and Vanhoutte 1985). In other experiments it was shown that upon exposure to radioactive 5-HT, transmural stimulation released labelled 5-HT; again the release was accompanied by a decrease in evoked noradrenaline release which was sensitive to methiothepin (Cohen 1987). Taken together, these findings are in favour of a physiological or pathophysiological role of platelet-derived 5-HT mediating prejunctional inhibition of sympathetic neurotransmitter release after being incorporated into and released from sympathetic nerves as a "cotransmitter". The endothelium, however, represents a considerable barrier for platelet-derived 5-HT to reach the noradrenergic nerves in the canine saphenous vein (Verbeuren et al. 1988). A further source for 5-HT may be mast cells. Interestingly, degranulation of mast cells either by compound 48/80 or by allergen challenge in the rat heart was accompanied by a release of not only histamine, but also 5-HT, and the evoked overflow of endogenous noradrenaline, investigated during mast cell degranulation, was inhibited in a methiotepin- and partly ketanserin-sensitive manner (Fuder et al. 1994). This indicates a prejunctional role of mast cell-derived 5-HT in the rat heart, which may become relevant under pathophysiological conditions involving mast cell degranulation.

2.6 Histamine Receptors

Histamine is known to modulate basal and stimulation-evoked release of noradrenaline (see Marshall 1981). Whereas histamine facilitates basal

overflow by pathways unrelated to histamine receptor activation (see e.g. Rand et al. 1982a), the stimulation-evoked overflow is in many instances inhibited in a histamine receptor-dependent manner (see Marshall 1981, Table 3). Only few reports exist that show an increase in sympathetic neurotransmitter release. Such an increase may be the result of a competition between histamine and noradrenaline at the uptake 1 carrier (Rand et al. 1982a), and as such does not represent a true facilitation, but merely a reduction of an elimination pathway for noradrenaline. Facilitation of evoked overflow sensitive to H_1 receptor blockade was found in goat cerebral arteries (Miranda et al. 1992), and indirect evidence for facilitation was also obtained in the rabbit kidney (Ercan and Türker 1981). Other observations in favour of an H_2 receptor-mediated facilitation were discussed to be non-specific in nature (Rand et al. 1982b). Interestingly, degranulation of mast cells in guinea-pig atria (Wong-Dusting et al. 1982) and rat heart (Ries and Fuder 1993, Fuder et al. 1994) resulted in an increase in evoked noradrenaline overflow. In the rat heart, the facilitation was blocked by cimetidine and was probably related to a postganglionic, prejunctional facilitatory effect of endogenous histamine via activation of H_2 (and not via H_1 or H_3) receptors (Fuder et al. 1994). Surprisingly little is known about the influence of endogenous histamine released upon degranulation of mast cells and about possible interactions between mast cells and sympathetic nerves. Facilitatory effects would not be surprising, since an excitatory effect of endogenous histamine released by allergen challenge has been shown in vitro by electrophysiological methods in superior cervical ganglia of guinea pigs sensitized to ovalbumin (Christian et al. 1989). It was also suggested that exogenous or endogenous histamine (released upon allergen challenge) may have inhibitory (H_3-mediated) or excitatory (H_1-mediated) effects in different synapses of the guinea-pig superior cervical ganglia (Christian and Weinreich 1992).

In contrast, the effects of exogenous histamine receptor agonists on noradrenaline release are well known. Whereas early reports were in favour of an H_2 or H_1 receptor-mediated inhibition (see Marshall 1981; Table 3), more recent results obtained after the discovery of H_3 receptors (see Haaksma et al. 1990) show that predominantly H_3 receptors are involved in the inhibition of sympathetic neurotransmission. The extent of inhibition usually does not exceed 40% of the evoked overflow, but is consistently seen under many conditions in vitro and in vivo in different species and organs (Table 3). It appears to involve a pertussis toxin-sensitive G protein (Nozaki and Sperelakis 1989). Some of the early findings, but perhaps not all, may be reinterpreted in view of a

Table 3. Modulation of sympathetic neurotransmitter overflow/neurotransmission by histamine receptors

Species	Tissue	Stimulus +parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Rat (pithed)	Heart	FS, 0.5 Hz increase in heart rate	2-(2-Thiazolyl)ethylamine, dimaprit, *R*-(−)-α-methylhistamine	Inhibition	Thioperamide	H_3	No evidence for prejunctional H_1 or H_2 receptors	Malinowska and Schlicker 1993
Rat	Heart	SNS, 3 Hz, 270 p. in trains of 6 p. NA	Endogenous histamine	Facilitation	Cimetidine	H_2	Histamine released by compound 48/80 and determined; facilitation partly opposed by methiothepin-sensitive inhibition due to 5-HT release (also determined)	Ries and Fuder 1993; Fuder et al. 1994
Rat (pithed)	Blood vessels	FS, 0.5 Hz Pressure	R-(−)-α-Methylhistamine	Inhibition	Dimetindene, ranitidine, thioperamide	H_3	Inhibition of pressure increase sensitive to thioperamide only	Malinowska and Schlicker 1991
Guinea pig	Nasal mucosa	FS, 10 Hz, 30 s ^{3}H-NA	Histamine	Inhibition	Cimetidine	H_2? (H_3?)	Inhibition sensitive to very high cimetidine concentrations; receptor type unclear	Kubo et al. 1989
Guinea pig	Atria	FS, 2 Hz, 10 s ^{3}H	Histamine	Inhibition	Mepyramine, cimetidine	unclear	Blockade of effects by both antagonists explained by "non-specific" effects; not mediated by muscarinic or cyclooxygenase-related mechanisms; independent of uptake 1; explained by non-selective effect or antagonist activity	Rand et al. 1982a
			Impromidine	Facilitation	Mepyramine, cimetidine	unclear		Rand et al. 1982b

Guinea pig	Atria	FS, 2 Hz, 10 s ^{3}H	Endogenous histamine	Inhibition Facilitation	Cimetidine	H? H?	Inhibition of evoked release upon allergen (low dose) challenge on ovalbumin-sensitized atria insensitive to cimetidine; facilitation by high allergen dose, mechanisms?	Wong-Dusting et al. 1982
Guinea pig	Atria	FS ^{3}H-NA and NA	Histamine, *R*(−)-α-methyl-histamine	Inhibition	Mepyramine, cimetidine, thioperamide	H_3	Similar inhibition by agonists for newly incorporated and endogenous NA	Fuder et al. 1990
Guinea pig	Atria	FS inotropy	R(−)-α-methyl-histamine	Inhibition	Thioperamide	H_3	Functional evidence for prejunctional inhibition	Luo et al. 1991
Guinea pig	Heart and circulation	FS (medulla oblongata), 34 Hz Increase in heart rate and blood pressure	R(−)-α-methyl-histamine	Inhibition	Thioperamide, impromidine, burimamide	H_{3B}	Subclassification according to rank order of antagonist potency (thioperamide = burimamide > impromidine)	Hey et al. 1992a
Guinea pig	Mesenteric artery	Focal FS e.j.p.	Histamine + 5 others	Inhibition	Impromidine + 5 others	H_3	Slight stimulatory effect of impromidine possibly indicating inhibition by histamine; histamine effect like that of NA PTT sensitive	Ishikawa and Sperelakis 1987 Nozaki and Sperelakis 1989
Rabbit	Kidney	SNS Increase in perfusion pressure	Histamine, 2-(2-Thiazolyl)ethylamine,	Facilitation	Mepyramine	H_1	Conclusions of prejunctional effects hampered by strong postjunctional effects; interpretation of blockade	Ercan and Türker 1981

Table 3 (*Contd.*)

Species	Tissue	Stimulus +parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
			dimaprit, impromidine	Inhibition	Metiamide	H_2? (H_3?)	by metiamide (H_2 and H_3 antagonist, see Haaksma et al. 1990) also compatible with H_3 receptors?	
Cat	Iris in vivo	SNS mydriasis	R(−)-α-methyl-histamine	Inhibition	Chlorphenir-amine, cimetidine, thioperamide	H_3	Presynaptic postganglionic site of interaction favoured, but ganglionic location of interaction not excluded	Koss and Hey 1993
Cat	Nictitat-ing mem-brane in vivo	SNS contraction	R(−)-α-methyl-histamine	Inhibition	Chlorphenir-amine, cimetidine, thioperamide	H_3	Site of action postganglionic prejunctional, no ganglionic interaction	Koss and Hey 1992
Dog	Heart in vivo	SNS, 3 Hz Increase in heart rate	Histamine	Inhibition	Chlorphenir-amine, metiamide, cimetidine	H_1? (H_3?)	Receptor type unclear since chlorpheniramine ($H_1$and H_3 antagonist; see Haaksma et al. 1990) only blocked inhibition	Kimura and Satoh 1983
Pig	Retina discs	FS, 3 Hz, 2 min ^3H	Histamine, 2-(2-thiaz-olyl)ethyl-amine, dimaprit, *R*(−)- and *S*(+)-α-methyl-histamine	Inhibition	Dimetindene, ranitidine, thioperamide	H_3	Uptake 1 and α_2-adrenocep-tors blocked	Schlicker et al. 1990

Goat	Cerebral arteries	FS, 8 Hz 300 p. ^3H or contraction	Histamine 2-pyridyl-ethylamine, dimaprit	Facilitation	Diphenhydramine, cimetidine	H_1	Increase in evoked overflow of tritium by histamine, and in evoked contractions (but not in the effect of exogenous NA) by histamine and the other agonists tested	Miranda et al. 1992
Cattle	Splenic vein	FS, 10 Hz, 300 p. ^3H	Histamine	Inhibition	Pyrilamine, cimetidine	H_1	Postjunctional contraction of histamine counteracts the prejunctional effect; inhibitory effect of high phentolamine concentrations	Dzielak et al. 1983
Man	Saphenous vein	FS, 2 Hz, 3 min ^3H	Histamine, 2-(2-thiazolyl) ethylamine, dimaprit, *R*(−)- and *S*(+)-α-methyl-histamine	Inhibition	Pheniramine, ranitidine, thioperamide	H_3	Histamine effect independent of α_2-adrenoceptor blockade; stereoselective effect; (+)-enantiomer of agonist inactive	Molderings et al. 1992a

For explanations, see Table 1.

possible weak (but sufficient) H_3 receptor blockade by some of the antagonists used which, at that time, were considered to be H_1 or H_2 selective (see Haaksma et al. 1990).

2.7 Imidazoline Receptors

The imidazoline derivative BDF 6143 (4-chloro-2-[2-imidazolin-2-ylamino]-isoindoline hydrochloride), an α_2-adrenoceptor antagonist, was shown to be capable not only of facilitating the evoked noradrenaline release in the presence of yohimbine or rauwolscine in rabbit pulmonary artery and aorta via blockade of α_2-adrenoceptors, but also of inhibiting it (Docherty et al. 1982). The mechanism of action remained obscure for many years. Recent work by Molderings et al. (1991) and Göthert and Molderings (1991) has shown that many imidazoline or guanidine derivatives, as well as phenethylamines to some extent, inhibit sympathetic neurotransmitter release by a mechanism independent of α_2-adrenoceptor activation. The inhibitory effect is observed with agonists and antagonists at α_2-adrenoceptors. It was postulated that the effect is mediated by an imidazoline/guanidine receptive site located prejunctionally on sympathetic nerves (Molderings et al. 1991; Göthert and Molderings 1991). This putative receptor is present on sympathetic nerves in rabbit blood vessels (see above) and hearts (Fuder and Schwarz 1993), possibly also on sensory nerves in the rabbit iris (Fuder and Selbach 1993), and can be blocked by rauwolscine with a very low affinity (about 50-fold less than that for α_2-adrenoceptors; pA_2 around 6.7; see Molderings et al. 1991, Fuder and Schwarz 1993) and by high concentrations (at a long exposure time) of phenoxybenzamine (Molderings et al. 1991).

The prejunctional "imidazoline receptor" was subject to desensitization upon prolonged exposure to high agonist concentrations (Fuder and Schwarz 1993). Surprisingly, attempts to desensitize selectively with BDF 6143 without simultaneously desensitizing prejunctional α_2-adrenoceptors failed and, under the conditions investigated, cross-desensitizations of both receptors were observed (Fuder and Schwarz 1993). This sheds some doubts on the hypothesis that α_2-adrenoceptors and imidazoline receptors are separate functional units. Nevertheless, the putative prejunctional imidazoline receptors can be clearly separated from the various types of imidazoline receptors characterized by functional experiments or by binding studies (see Michel and Ernsberger 1992, Hieble and Ruffolo 1992). The relative potency of agonists (see

Molderings et al. 1991, Göthert and Molderings 1991, Fuder and Schwarz 1993) and the sensitivity to rauwolscine discriminates the prejunctional putative imidazoline receptor from the other imidazoline sites (I_1 or I_2 sites and subgroups; Michel and Ernsberger 1992). It is unclear whether an endogenous agonist exists for the putative prejunctional imidazoline receptor and whether it is involved in effects of imidazoline or guanidine derivatives in vivo. If at all, an imidazoline receptor agonist has a chance of inhibiting noradrenaline release only under conditions of small or lacking α_2-adrenoceptor-mediated autoinhibition (see Göthert and Molderings 1991).

2.8 Adenosine Receptors/Purinoceptors

Adenosine receptors are widely found on peripheral sympathetic nerves in all species so far investigated (including cats) and their function has been comprehensively reviewed previously (see Starke 1977; Vanhoutte et al. 1981; Su 1983; Fredholm and Dunwiddie 1988; Olsson and Pearson 1990). Only recently was heterogeneity in the function of adenosine receptors and receptor subtypes detected in the peripheral sympathetic nervous system. Thus, besides inhibitory actions, facilitation of evoked noradrenaline release were seen in peripheral tissues (see Table 4). Whereas inhibition usually involves A_1 adenosine receptors (see reviews quoted above and Table 4; for an exception in the dog in vivo where A_1 receptors mediate a facilitation of *afferent* sympathetic nerve activity, see Dibner-Dunlap et al. 1993), facilitation observed in rat iris and vas deferens is brought about by A_2 receptors (Fuder et al. 1992; Gonçalves and Queiroz 1993). In the rat iris, endogenous purinoceptor agonists mediate simultaneously modest inhibition and small facilitation as evident from a decrease by an A_2 receptor antagonist (in the absence and presence of an A_1 antagonist) and an increase by an A_1 selective antagonist of stimulation-evoked overflow of radioactive authentic noradrenaline (Fuder et al. 1992). Little is known about the functional or physiological relevance of the purinergic A_2 receptor-mediated facilitation.

In contrast, the inhibition by endogenous purines via A_1 receptor activation is well characterized. It has been suggested to be of minor importance in normoxic conditions and upon stimulation with short trains of 5 Hz up to 30 s (Wennmalm et al. 1988). The endogenous purines may mediate a strong influence in hypoxia and upon prolonged nerve activation (see Fredholm and Dunwiddie 1988). Results recently obtained in the rat perfused heart which show an increased adenosine

Table 4. Modulation of sympathetic neurotransmitter overflow/neurotransmission by adenosine receptors/purinoceptors

Species	Tissue	Stimulus +parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Rat	Iris	FS, 3 Hz, 2 min ^{3}H-NA	CPA, *R*(−)-PIA, CHA, NECA, adenosine	Inhibition	8-PT, DPCPX	A1	Endogenous agonists mediate inhibition (by A_1 and P_{2Y}-like receptors) and facilitation (by A_2 receptors); adenosine (but not ATP) induced inhibition	Fuder et al. 1992
				Facilitation	CP-66713,	A_2		
			ATP	Inhibition	cibacron blue, suramin	P_{2Y}	blocked by adenosine deaminase; uptake 1 and 2 and α_2-adrenoceptors blocked	Fuder and Muth 1993
Rat	Heart	SNS, 4 Hz, 0.5–1 min NA	CHA, *R*(−)-PIA, *S*(+)-PIA adenosine	Inhibition	8-PT	A_1	Under hypoxic conditions adenosine outflow was increased and accompanied by an 8-PT sensitive inhibition	Richardt et al. 1987
Rat	Portal vein	FS, 1 or 5 Hz ^{3}H	Adenosine	Inhibition	Aminophylline	A	Inhibition of adenosine deaminase reduced, and antagonist increased evoked overflow indicating endogenous purinergic tone; release of purines of pre- and postjunctional origin determined	Enero 1981
Rat	Mesentery	SNS, 7 Hz, 30 min NA	CADO	Inhibition	DPSPX	A_1	Lack of tone by endogenous purines despite evidence for large reduction of tissue perfusion	Kuan and Jackson 1988
Rat	Stomach	SNS, 1 Hz, 1 min NA	Endogenous agonist	Inhibition	8-SPT	A	Methoxamine induced inhibition probably mediated by adenosine/purine released upon stimulation of postjunctional α_1-adrenoceptors	Yokotani et al. 1992

Rat	Tail artery	FS, 20 Hz, 10–50 p. Contraction	Endogenous agonist	Inhibition	α,β-meATP	P_2	Autoinhibitory function indirectly shown by increase of response to NA release in the presence of α,β-meATP	Bao et al. 1989
Rat	Caudal artery	FS, 1 Hz, 3 min NA	CADO, adenosine, ATP	Inhibition	8-SPT	P_3? (A_1 +P_2?)	Postulation of a novel type of receptor; methoxamine induced α_1-adrenoceptor mediated inhibition probably due to purine release	Shinozuka et al. 1988; Shinozuka et al. 1991
Rat	Caudal artery	FS, 0.4 Hz, 1 min ^{3}H	*R*(−)-PIA	Inhibition	8-PT	A_1	Vasoconstrictor response to FS less inhibited by endogenous adenosine receptor agonists in SHR compared to WKY rats	Illes et al. 1989
Rat	Vas deferens	FS, 2 Hz, 3 min NA	CADO, adenosine, ATP, β,γ-meATP, UTP	Inhibition	8-SPT (α,β-meATP)	P_3? (A_1 +P_2?)	Adenosine uptake inhibitor increased, adenosine deaminase decreased, the adenosine induced inhibition; P_3 receptor postulated, but for alternative explanation see Kurz et al. 1993	Forsyth et al. 1991
Rat	Vas deferens	FS, 1 Hz, 1 min ^{3}H	CADO, β,γ-me-ATP, α,β-me-ATP, 2-mcSATP	Inhibition	DPCPX, suramin	A_1 P_2	DPCPX antagonized the effects of CADO and of β,γ-meATP, but not suramin	Kurz et al. 1993
Rat	Vas deferens	FS, 6p., 100 Hz, 60 p., 1 or 8 Hz, ^{3}H			DPCPX, suramin, reactive blue 2	A_1 P_{2y}-like	Uptake 1 and 2 and α_2-adrenoceptors blocked; suramin induced increase in evoked overflow PTX sensitive	von Kügelgen et al. 1994

Table 4 (*Contd.*)

Species	Tissue	Stimulus +parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Rat	Vas deferens	FS, 5 Hz, 9 min NA	NECA, *R*(−)-PIA, CGS21680	Inhibition Facilitation	DPCPX, DMPX	A1 A_{2A}	Uptake 1 and 2 and MAO blocked; facilitatory and inhibitory tone by endogenous agonists, affected by adenosine uptake inhibitor	Gonçalves and Queiroz 1993
Mouse	Vas deferens	FS, 5 Hz, 50 (or 5) p. NA (or e.j.p.)	CADO, CHA, *R*(+)-, S(+)-PIA, NECA	Inhibition	8-PT	A_1	No indication for inhibitory tone by endogenous purines	Blakeley et al. 1988
Mouse	Vas deferens	FS, 2 Hz, 20 p. ^3H	ATPγS, ATP, adenosine, UTP, α,β-meATP	Inhibition	8-SPT	A_1 P_{2Y}-like	8-SPT blocked adenosine and UTP effect but not that of ATP and ATPγS; no desensitization by α,β-meATP of adenosine-induced inhibition	von Kügelgen et al. 1989
Mouse	Vas deferens	FS, 1 Hz, 1 min ^3H	CADO, β,γ-meATP, α,β-meATP, 2-meSATP, ATPγS	Inhibition	DPCPX, suramin	A_1 P_2	DPCPX antagonized the effects of CADO, β,γ- and a,β-meATP; suramin reduced that of β,γ-meATP, but not of CADO; autoreceptor-like function of P_2 receptors	Kurz et al. 1993
		FS, 8 Hz ^3H			Reactive blue 2, coomassie blue, PPADS	P_{2Y}-like		von Kügelgen et al. 1994
Guinea pig	Heart	SNS, 12 Hz, 1 min NA	CHA	Inhibition		A	Evoked NA and neuropeptide Y overflow sensitive to inhibition	Haass et al. 1989
Guinea pig	Heart papillary	FS, 3 Hz, 2 min	CPA, *R*(−)-PIA,	Inhibition	DPCPX	A_1	Low affinity of DPCPX despite little inhibition by	Schütz et al. 1991

	muscle	^3H	NECA				endogenous purines; PTX-insensitive	
Guinea pig	Pulmonary artery	FS, 5 Hz, 50 s ^3H	*R*(−)-PIA, adenosine, NECA	Inhibition	8-SPT	A_1	In addition to inhibition of evoked overflow, increase in total overflow was observed	Wiklund et al. 1989b
Guinea pig	Saphenous artery	Focal FS, 0.5 Hz e.j.p.	Endogenous purines	Inhibition	$ANAPP_3$	P_2	$ANAPP_3$ induced increase in response to transmitter release taken as evidence for purinergic "autoinhibition"	Fujioka and Cheung 1987
Guinea pig	Vas deferens	FS, 8 Hz, 300 p. ^3H	Endogenous purines	Inhibition	α,β-meATP	P_2	α,β-me-ATP induced increase in NA release taken as evidence for purinergic "autoinhibition"	Stjärne and Åstrand 1985
Rabbit	Heart	SNS, 5 Hz, 30 s NA	Adenosine	Inhibition	Theophylline, 8-SPT	A	Lack of effect of endogenous purines under normoxic conditions, inhibition under hypoxia	Wennmalm et al. 1988 Wennmalm 1988
Rabbit	Ear artery	FS,10 Hz NA	ATP, AMP-PNP	Facilitation	α,β-meATP	P_2	Facilitatory effect of ATP reduced by α,β-meATP.	Miyahara and Suzuki 1987
		FS, 4 Hz, 3 min NA	ATP		Suramin, α,β-meATPP		Suramin- and prazosin-sensitive facilitation by methoxamine accompanied by increase in endogenous ATP overflow	Ishii et al. 1993
Rabbit	Mesenteric artery	Focal NS, 1 Hz, 15 p. e.j.p	*R*(−)-PIA, NECA, S(+)-PIA, adenosine	Inhibition	8-PT, 8-CPT, 8-SPT	A_1	Sympathetic cotransmitter ATP probably responsible for e.j.p.; adenosine effect enhanced by uptake inhibitor, decreased by adenosine deaminase	Illes et al. 1988

Table 4 (*Contd.*)

Species	Tissue	Stimulus +parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Cat	Cerebral artery	FS, 8 Hz, 1 min, or 50 m*M* K^+ ^3H	Adenosine *R*(−)-, *S*(+)-PIA, NECA, ATP, AMP	Inhibition		A_1	Inhibitory effect of adenosine uptake inhibitor indicates prejunctional function of endogenous purines	Rivilla et al. 1990
Cat	Femoral artery	FS or 50 m*M* K^+ ^3H	Adenosine, AMP, ATP, NECA, *R*(−)-PIA	Inhibition	Methyl-isobutyl-xanthine	A_1/A_2	Small inhibitory tone by endogenous purines in the absence and presence of adenosine uptake inhibition	Güemes et al. 1990
Dog	Kidney in vivo	SNS, 1–3 Hz, 0.5–1.5 min Renal NA release rate	Adenosine	Inhibition	Theophylline	A	Prejunctional inhibition offset by postjunctional facilitation of response to NA release; prejunctional effect of endogenous purinoceptor agonist observed	Yoneda et al. 1990
Man	Fallopian tube	FS, 5 Hz, 0.5 min ^3H	Adenosine, *R*(−)-PIA, NECA	Inhibition	8-SPT	A_1	Prejunctional inhibition counteracted by stimulatory postjunctional A_1 receptors increasing response to NA release; additionally postjunctional inhibitory A_2 receptors present	Wiklund et al. 1986

For explanations and codes, see Table 1.

α,β-meATP or β,γ-meATP, α,β-methylene-ATP or β,γ-methylene-ATP; 2-meSATP, 2-methylthio-ATP; 8-CPT or 8-PT, 8-cyclopentyltheophylline or 8-phenyltheophylline; 8-SPT, 8-p-sulphophenyltheophylline; AMP-PNP, 5′-adenylylimidodiphosphate; $ANAPP_3$, arylazidoaminopropionyl-ATP; ATPγS, adenosine-5′-O-(3-thio)-triphosphate; CADO, 2-chlororadenosine; CHA or CPA, N^6-(cyclohexyl) adenosine or N^6-(cyclopentyl) adenosine; DMPX, 3,7-dimethyl-1-(2-propynyl) xanthine; DPCPX or DPSPX, 1,3-dipropyl-8-cyclopentylxanthine or 1,3-dipropyl-8-p-sulfophenylxanthine; NECA, 5′-N-ethylcarboxamidoadenosine; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; *R*(−)-PIA or *S*(+)-PIA, *R*(−)- or *S*(+)-N^6-(2-phenylisopropyl)-adenosine

outflow and a purinoceptor antagonist-sensitive decrease in evoked noradrenaline release under hypoxic conditions compared with normoxia confirm this view (Richardt et al. 1987). Very often, adenosine receptor antagonists failed to increase the evoked overflow or enhanced it only to a small degree. In case of non-selective (A_1 plus A_2) antagonists, however, a lack of effect on evoked overflow may not be taken as evidence for a lack of influence by endogenous purines (nucleosides or nucleotides), since concomitant inhibitory (A_1) and facilitatory (A_2) influences may be blocked simultaneously, resulting in unchanged release of neurotransmitter. Thus, 8-phenyltheophylline failed to modify evoked noradrenaline overflow in rat iris, but DPCPX (A_1-selective) increased and CP-66713 (A_2-selective) decreased it (Fuder et al. 1992).

Not only adenosine but also ATP and related nucleotides are present in the synaptic cleft (see Starke 1977, Su 1983, Burnstock 1990, von Kügelgen and Starke 1991b). ATP functions as a cotransmitter and is released from adrenergic nerves upon exocytosis (see Burnstock 1990, von Kügelgen and Starke 1991b, Kupfermann 1991). In different tissues, varying proportions of the synaptic ATP (20%–97%, see Su 1983, von Kügelgen and Starke 1991b) originate from postjunctional sites upon α_1-adrenoceptor stimulation by noradrenaline. ATP is quickly metabolized to adenosine (for review see Su 1983). Both ATP or related compounds and adenosine or other adenosine receptor agonists may stimulate prejunctional adenosine receptors (e.g. Kurz et al. 1993).

While it used to be justified to express considerable doubt about ATP acting via a separate purinoceptor on the prejunctional site (e.g. Su 1983, Olsson and Pearson 1990), there is now solid evidence for a neuromodulatory role of nucleotides via P_2 receptors (Table 4). Evidence comes from studies with exogenous purinoceptor agonists which inhibit evoked noradrenaline release to a comparable extent in the absence and presence of adenosine receptor antagonists or are affected by these blockers to a small extent only, not compatible with an interaction at adenosine receptors (Fuder et al. 1992; Fuder and Muth 1993; Kurz et al. 1993). In contrast, P_2 receptor antagonists such as cibacron blue (reactive blue 2) inhibit the effects of the P_2 receptor agonists, but not of adenosine receptor agonists (Fuder and Muth 1993; Kurz et al. 1993). Moreover ATP, unlike adenosine, inhibits evoked sympathetic neurotransmitter release in the presence of adenosine deaminase (Fuder et al. 1992). P_2 receptor antagonists may even increase the evoked noradrenaline overflow, indicating an "autoinhibitory" effect of endogenous nucleotides. This was investigated in the rat iris in the presence of an A_1 receptor antagonist by Fuder and Muth (1993) and in the mouse

and rat vas deferens by von Kügelgen et al. (1993a, 1994). The P_2 purinoceptor-mediated autoinhibition increases with the train length and frequency of stimulation (von Kügelgen et al. 1994). So far, potent and subtype-selective P_2 antagonists are not available, and caution is warranted in interpreting the findings with regard to subclassification of the prejunctional P_2 purinoceptor. But clearly, the coexistence of A_1 adenosine receptors and P_2 purinoceptors (probably of the P_{2Y} subtype; see Table 4) in tissues of the rat also explains previous findings which led to the postulation of a new subtype of purinoceptor, the P_3 receptor (see Shinozuka et al. 1988, Forsyth et al. 1991). As a rule, P_2 receptors inhibit the evoked noradrenaline release, but a well-documented exception appears to be the rabbit ear artery in which a facilitatory effect was observed (Miyahara and Suzuki 1987; Ishii et al. 1993). Whereas there is still debate as to whether a presumed "A_3 receptor" (not identical with the novel A_3 receptor, see below) has a prejunctional function, a functional postjunctional role of a novel A_3 receptor (Fozard and Carruthers 1993) has been established (see Collis and Hourani 1993).

2.9 Prostanoid and Leukotriene Receptors

The prejunctional modulation of noradrenaline release by endogenous and exogenous prostanoids has been reviewed before (Hedqvist 1977; Starke 1977; Vanhoutte et al. 1981; Güllner 1983; Rand et al. 1990). Endogenous prostanoids are formed in response to noradrenaline release or are partly of prejunctional origin. Only little is known about the prejunctional effects of metabolites of arachidonic acid produced by the 5-lipoxygenase pathway (see Feuerstein 1985). Recently, a facilitatory effect of the cyclooxygenase inhibitor indomethacin was discussed as involving not only the abolition of an inhibitory effect of endogenous prostanoids but possibly in addition a facilitation due to enhanced production of endogenous arachidonic acid metabolites of the 5-lipoxygenase pathway (Racké et al. 1992a).

Whereas leukotriene-mediated prejunctional effects are thus not firmly established yet, prostanoid-mediated modulation of release is well characterized and represents an example of transsynaptic modulation of neurotransmission. Such modulation may represent an important step in regulation of release induced by other drugs such as bradykinin: Bradykinin enhanced the outflow of prostanoids, and the bradykinin-mediated inhibition of noradrenaline release in the rabbit pulmonary

artery and heart was sensitive to indomethacin (Starke et al. 1977). It was concluded that the bradykinin effect was indirect and probably involved the formation of prostanoids of the E series.

Only few prejunctional prostanoid and thromboxane receptors have been subclassified according to recent classification schemes (see Table 5). Lack of a variety of subtype selective antagonists (which became available only recently) and insufficient agonist specificity for subtypes have so far hampered an unequivocal receptor classification. Receptors in rat trachea (Racké et al. 1992a) and vena cava (Molderings et al. 1992b) and rabbit (Ohia and Jumblatt 1990a) or human iris ciliary body (Ohia and Jumblatt 1991) which were so far classified belong to the EP_3 subtype. There is no doubt, however, that in many tissues inhibition of cyclooxygenase leads to a modest increase or decrease in evoked noradrenaline overflow under various conditions, indicating a physiological role of prostaglandin (PG), prostacyclin or thromboxane-mediated modulation of release (inhibition or facilitation; see Table 5). In some tissues, prejunctional modulation of release is accompanied by a corresponding modification of response to neurotransmitter release (e.g. rabbit portal vein, dog mesenteric artery); but in other cases (e.g. human vas deferens, rat kidney) additional postjunctional effects act in the opposite direction to the expected modification of response according to the prejunctional effect. Thus despite modification of neurotransmitter release, neurotransmission (which involves both the prejunctional and postjunctional response) may not necessarily be altered, or the opposite postjunctional effect may even represent the predominant action component. The prejunctional inhibition of noradrenaline release by endogenous PGs in rat kidney cortex was pertussis toxin insensitive (Murphy and Majewski 1990b). In contrast, pertussis toxin blocked (at least partly) the inhibition of Ca^{2+} channels by prostaglandin E_2 (PGE_2) in rat sympathetic neurones (Ikeda 1992).

2.10 Amino Acid Receptors

γ-Aminobutyric acid (GABA) is an important inhibitory neurotransmitter/neuromodulator in the central nervous system. Whereas the functional role of GABA appears to be confined to the central nervous system, peripheral nerves are nevertheless endowed with prejunctional GABA receptors (for recent comprehensive reviews, see Erdö and Bowery 1986 and Bowery 1993). There is no firm evidence for a modulatory role of $GABA_A$ receptors on sympathetic nerves. The L-alanine

Table 5. Modulation of sympathetic neurotransmitter overflow/neurotransmission by prostanoid or leukotriene receptors

Species	Tissue	Stimulus +parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Rat	Trachea	FS, 3 Hz, 540 p. NA	Nocloprost, sulprostone, PGE_2, iloprost, U46619	Inhibition	AH6809	EP_3	Uptake 1 and α_2-adrenoceptors blocked; inactivity of AH-6809 (EP_1 antagonist) and relative agonist potency suggests EP_3 subtype; facilitation by indomethacin ("epithelium" dependent) perhaps not only due to blockade of prostanoid synthesis, but also to increased leukotriene synthesis	Racké et al. 1992a
Rat	Heart	SNS, 3Hz, 1 min or K^+ 60 μmol ^3H	PGE_2, PGI_2	Inhibition		PG	Indomethacin increased evoked overflow indicating endogenous tone and reduced inhibitory effect of arachidonic acid	Khan and Malic 1982
Rat	Vena cava	FS, 0.66 Hz, 6 min ^3H	PGE_2, misoprostol, sulprostone, PGE_1, PGE_2, iloprost, $PGF_{2\alpha}$, PGD_2, U46619	Inhibition	AH6809	EP_3	Uptake 1, 2 and in some experiments α_2-adrenoceptors blocked; subtype suggested according to relative potency and inactivity of antagonist; no tone by endogenous agonist; no interaction between α_2-adrenoceptors and EP_3 receptors	Molderings et al. 1992b
Rat	Kidney	SNS, 1 Hz, 30 s ^3H	PGE_2, PGI_2, $PGF_{2\alpha}$, iloprost, U46619	Inhibition	Daltroban	PG	Only PGE_2 active as prejunctional inhibitor; strong postjunctional facilitatory effects of most agonists; postjunctional effect of U46619	Rump and Schollmeyer 1989

							(TxA_2 agonist) daltroban (TxA_2 antagonist) sensitive	
Guinea pig	Atria	FS, 4 Hz, 2–12 p., or 4 Hz, 100 p. contraction or ^3H	TxB_2, ONO11006, U46619	Inhibition	Sulotroban	Tx	No effect on ^3H-release but inhibition of postjunctional responses to FS (and not to exogenous NA); discrepancy perhaps due to different stimulation parameters	Mantelli et al. 1992
Guinea pig	Pulmonary artery	FS, 5 Hz, 100 p. ^3H	PGE_1, PGE_2, PGI_2, $PGF_{2\alpha}$	Inhibition	Diphloretin phosphate	PG	Inhibition by PGs of the E series only; small inhibitory tone by endogenous PGs in presence of arachidonic acid	Yasuda and Misu 1988
Guinea pig	Vas deferens	FS, 2 or 20 Hz, 240 p. ^3H	PGE_2	Inhibition		PG	Evoked NA release inhibited, but ATP release enhanced by PGE_2 (only at 2, not at 20 Hz)	Ellis and Burnstock 1990
Rabbit	Iris ciliary body	FS, 5 Hz, 1 min ^3H	Sulprostone, 16,16-dime-PGE_2, PGE_2, 11-deoxy-PGE_1, iloprost, $PGF_{2\alpha}$, U46619	Inhibition	AH6809	EP_3	No tone by endogenous prostanoids or leukotrienes (revealed by lack of influence of indomethacin or lipoxygenase inhibitor); no effect by Tx agonist	Ohia and Jumblatt 1990a
Rabbit	Heart	SNS, 5 Hz, 30 s NA					No effect of indomethacin on evoked overflow despite >90% reduction of prostanoid efflux, not even under hypoxia; for opposite findings see Hedqvist 1987 and Wennmalm et al. 1987	Wennmalm 1988
Rabbit	Vas deferens	FS, 5 Hz, 2 min ^3H	Endogenous PG	Inhibition		PG	Uptake 1 and 2 blocked; inhibition by angiotensin III of evoked release sensitive to indomethacin, hence perhaps due to formation of PG	Saye et al. 1986

Table 5 (*Contd.*)

Species	Tissue	Stimulus +parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Rabbit	Portal vein	FS, 4 Hz, 30 s NA	TxA_2	Facilitation	SQ30741	Tx	Uptake 1 and 2 blocked; platelet microsomes incubated in arachidonic acid form TxA_2 (detected upon metabolism as TxB_2) which causes facilitation of release and potentiation of twitch response	Trachte and Stein 1988
Rabbit	Oviduct	FS, 1 Hz, 300 p. ^3H	PGE_2	Inhibition		PG	Uptake 1 and 2 blocked inhibitory tone by endogenous PG revealed by indomethacin; PGE_2 effect more pronounced in oviduct of estradiol treated animals	Chernaeva and Charakchieva 1991
Dog	Mesenteric artery	FS, 5 Hz, 3 min ^3H	$PGF_{2\alpha}$ TxB_2	Facilitation	Diphloretin phosphate	PG Tx	Prejunctional facilitation or release reflected also in potentiation of contraction response; insensitive to diphloretin phosphate (in contrast to postjunctional effects of other PGs tested)	Nakajima and Toda 1986 Okamura et al. 1988
Dog	Mesenteric vein	FS, 0.5–5 Hz contraction	PGI_2	Facilitation ?		PG	Prejunctional facilitation indirectly concluded; interpretation complicated by interference between pre- and postjunctional effects	Herman et al. 1978

Man	Iris ciliary body	FS, 5 Hz, 1 min ^3H	Sulprostone, 16,16-dime-PGE_2, 11-deoxy-PGE_1, $PGF_{2\alpha}$, PGE_2	Inhibition	AH6809	EP_3	Receptor subtype concluded from relative agonist potency and inactivity of antagonist	Ohia and Jumblatt 1991 Jumblatt et al. 1993
Man	Vas deferens	FS, 5 Hz, 1 min ^3H (^3H-NA)	PGE_1, PGE_2, U44069, BM13505	Inhibition Facilitation		PG Tx	Opposite pre- and posjunctional effects of prostanoids interfering with each other	Holmquist et al. 1991

For explanations and codes, see Table 1.
PG, prostaglandin; Tx, thromboxane; 16,16-dime-PGE_2, 16,16-dimethyl prostaglandin E_2.

induced inhibition of [^{3}H]-noradrenaline release on rat atria, which was reduced by the $GABA_A$ receptor antagonist bicuculline (and strychnine), was suggested to involve $GABA_A$ (and glycine) receptors (Butta and Adler-Graschinsky 1987). In many tissues, however, $GABA_B$ receptors inhibit the stimulation-evoked overflow of noradrenaline in vitro and in vivo. In vitro this has been discussed for the rabbit pulmonary artery by Starke and Weitzell (1980), for the rat atria and mouse vas deferens (twitch) by Bowery et al. (1981) and Hill and Bowery (1981), and for the rat atria and guinea-pig vas deferens by Bowery et al. (1981), for the sympathetic response in bovine ovarian follicles by Kannisto et al. (1986), for the rat vena cava by Schneider et al. (1991) and Schlicker et al. (1993), for the sympathetic response in rat mesentery by Li and Duckles (1991), for the rat anococcygeus by Bowery (1993), for the middle cerebral artery of the goat by Miranda et al. (1989), and for the pig retina by Schlicker et al. (1993). In vivo studies have been carried out on pithed rat vasculature by Kohlenbach and Schlicker (1990) and Schlicker et al. (1993). Despite the presence of GABA in peripheral tissues, sympathetic neurotransmission was not affected by $GABA_B$ receptor antagonists. A physiological role of GABA acting via these peripheral prejunctional receptors is therefore rather doubtful, although it is not excluded that under some conditions GABA biophase concentrations that are sufficient to stimulate the receptors may be reached. Novel antagonists are now available which may lead to an extension of the $GABA_B$ receptor subclassification into four subtypes proposed for transmitter release-modulating receptors on synaptosomes of the central nervous system (Bonanno and Raiteri 1993).

Glutamate is one of the potent excitatory neurotransmitters in the central nervous system (see Ruzicka and Jhamandas 1993). To our knowledge, a glutamate receptor-mediated modulation of sympathetic neurotransmission in peripheral tissue has not been investigated. In contrast, there is evidence that noradrenaline release in the central nervous system is reduced by glutamate receptors of both the non-N-methyl-D-aspartate (NMDA) type and the NMDA type (Wang et al. 1992). In the rabbit brain cortex slices, the inhibition of electrically evoked [^{3}H]-noradrenaline release induced by ionotropic glutamate receptors [α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate and NMDA receptors] probably involved release of adenosine and adenosine receptor activation as an intermediate step (von Kügelgen et al. 1993b).

2.11 Nitric Oxide, Endothelin Receptors, and Epithelial Factors

Mediators such as EDRF (here used synonymously with nitric oxide, NO, for simplicity's sake; tools used in investigations on NO are mentioned in Sect. 3.9) or endothelin which are thought to be derived in part from endothelial sources have only recently attracted the interest of investigators in the prejunctional field. In the intact isolated perfused Langendorff heart of the rabbit, a system highly dependent on the coronary flow, EDRF and endothelin modified the outflow of noradrenaline in close correlation with changes in coronary flow rate; it was concluded that none of the drugs interfered with the process of sympathetic transmitter release directly but rather by changing the washout of released noradrenaline (Wennmalm et al. 1989). Changes in flow rate are accompanied by corresponding changes in mediator (EDRF, prostacyclin, and endothelin) formation (Wennmalm et al. 1991). Reduction in tissue perfusion may reduce the overflow of noradrenaline (due to enhanced elimination via uptake mechanisms), and inhibition of NO synthase is often accompanied by an increase in perfusion pressure and vascular resistance. Therefore, caution is warranted in interpreting the inhibition of evoked noradrenaline overflow by NO synthase inhibitors as an indication that NO directly influences exocytosis. In rat perfused mesentery, inhibition of NO synthase led to a decrease in stimulation-evoked noradrenaline release investigated in the absence of uptake inhibitors, and this was taken as evidence of a facilitatory prejunctional NO effect (Yamamoto et al. 1993). The findings could also be explained by reduction of tissue perfusion by NO synthase inhibition followed by an enhanced noradrenaline elimination in the tissue.

Overall there is only little evidence for a direct prejunctional effect of NO (EDRF) from different sources on sympathetic nerves, but plenty of data show a postjunctional influence on neurotransmitter responses. In isolated incubated segments of the rabbit carotid artery, endothelial factors released from a donor artery inhibited the contractions evoked by field stimulation of a bioassay artery, but failed to inhibit noradrenaline release (Cohen and Weisbrod 1988). It was concluded in this paper, that the endothelium may (i) metabolize noradrenaline, (ii) act as a physical barrier (see also Verbeuren et al. 1988) to the transmitter overflow into the blood vessel lumen, and (iii) inhibit sympathetic neurotransmission on a postjunctional level via EDRF, and perhaps on a prejunctional level via an additional effect independent of EDRF, prostanoid synthesis, free radicals and guanylyl cyclase. The inhibition

appears to involve voltage-dependent calcium channels (Tesfamariam et al. 1989). On isolated dog mesenteric arteries, EDRF (NO derived from endothelium) failed to affect evoked [^{3}H]-noradrenaline overflow even in the presence of superoxide dismutase (Toda et al. 1990). Whereas a postjunctional attenuation of responses to endogenous and exogenous noradrenaline was noticed, no evidence of a prejunctional effect of NO (or of activation of guanylyl cyclase by other drugs) was observed in the rat tail artery (Bucher et al. 1992a). Similarly, only a postjunctional increase in neuroeffector transmission, but no direct prejunctional effect on noradrenaline release was found after inhibition of NO synthase in guinea-pig pulmonary artery (Cederqvist et al. 1991). Interestingly, the increase outlasted the removal of the endothelium, rendering other tissue components likely candidates for sources of NO (for a discussion of neuronal NO as a neurotransmitter see Sect. 3.9). Neuronal NO, which functions as a non-adrenergic, non-cholinergic neurotransmitter in the rat isolated anococcygeus muscle, however, had no prejunctional influence on evoked [^{3}H]-noradrenaline release in that tissue, but inhibition of NO-synthase potentiated sympathetic neurotransmission at the postjunctional level by blocking the NO-mediated muscle relaxation (Brave et al. 1993). Taken together, NO from different tissue sources (endothelial, neuronal) may well have an inhibitory influence on sympathetic neurotransmission, but most likely via attenuation of postjunctional responses to noradrenaline release only. On the other hand, prejunctional inhibition by NO would not be surprising, since it was shown that activators of guanylyl cyclase reduce the evoked release of noradrenaline (Greenberg et al. 1990). Manipulation of the intraneuronal cyclic guanosine monophosphate (cGMP) content, however, is not necessarily accompanied by a modulation of sympathetic neurotransmission, determined by the overflow of endogenous noradrenaline from the rabbit carotid artery (Tesfamariam et al. 1992).

Endothelin may cause long-lasting increases in vascular tone, and suggestions about an influence on neurotransmitter overflow (and not on release itself) also apply to endothelin in perfused organs when noradrenaline uptake mechanisms are not blocked. Thus inhibitions by endothelin of evoked noradrenaline overflow in rat mesenteric artery (Tabuchi et al. 1989) and in rabbit (Wiklund et al. 1991) and dog (Takagi et al. 1991) kidneys do not constitute definite proof of a true prejunctional effect, since noradrenaline uptake was not blocked. No prejunctional effect was found in guinea-pig trachea (with functional responses to noradrenaline release in the presence of endothelin-1 and -3) and in pig kidney in vivo (Pernow and Lundberg 1989). In rat and guinea-pig

vas deferens (Wiklund et al. 1990) and guinea-pig pulmonary artery (Wiklund et al. 1989a), endothelin inhibited the evoked [^{3}H]-noradrenaline overflow. Both in guinea-pig pulmonary artery and in rat and guinea-pig vas deferens, the postjunctional response to noradrenaline release was enhanced by endothelin-1 (and endothelin-3) despite a reduction in transmitter overflow (induced by endothelin-1 only; Wiklund et al. 1989a, 1990), indicating a postjunctional facilitation of neurotransmission. In the rat vas deferens, the receptor subtype responsible for the facilitation appears to be an ET_A receptor (Warner et al. 1993), whereas the type of the inhibitory prejunctional receptor is unclear but seems to be different from that mediating the postjunctional response. Recent findings demonstrate that low endothelin-1 concentrations may facilitate evoked [^{3}H]-noradrenaline outflow and the postjunctional response to neurotransmitter release in the rat tail artery (Bucher et al. 1991). Thus no definite conclusions about the functional role of endothelin on neurotransmission and its physiological importance can be drawn.

Bronchial epithelial cells may be another source of factors with a prejunctional influence on noradrenergic nerves. In isolated rat trachea, removal of the epithelium was accompanied by a 30% reduction in tissue noradrenaline content, but a large decrease (up to 80%; exceeding that expected to be due to reduced transmitter content) in evoked noradrenaline overflow was observed, indicating possibly that epithelium-derived substances may have a permissive effect on noradrenaline release or suppress inhibitory factors (Racké et al. 1991). An epithelium derived relaxing factor facilitating β-adrenoceptor-mediated bronchodilation (or functionally inhibiting bronchoconstriction to acetylcholine, histamine and 5-HT) may facilitate sympathetic neurotransmission on a postjunctional level (see papers cited in Busk et al. 1990). Whether these processes have a physiological or pathophysiological role in asthma, where epithelium damage is often observed, is unclear.

2.12 Atrial Natriuretic Factor

Atrial natriuretic factor (ANF) is a tissue hormone that exerts a variety of postjunctional effects and is probably involved in a number of (patho-) physiological functions (Ruskoaho 1992). Its release from cardiac tissue is affected by the activation of autonomic nerves (see Ruskoaho 1992). It has been suggested that ANF inhibits sympathetic neurotransmission (see Kuchel et al. 1987). ANF inhibited the release of [^{3}H]-noradrenaline evoked by electrical stimulation in rat perfused

mesenteric arteries in vitro (Nakamura and Inagami 1986). ANF infused into pithed rats reduced elevations of plasma noradrenaline levels evoked by sympathetic stimulation without altering the clearance of noradrenaline or responses to exogenous noradrenaline (Zukowska-Grojec et al. 1986). More direct and indirect evidence has now been accumulated which demonstrates a prejunctional effect of exogenous ANF in various organs. Thus in the rabbit vas deferens, phasic contraction to field stimulation (representing a mainly purinergic response) and evoked noradrenaline release were inhibited by low concentrations of ANF (Drewett et al. 1989). Neither indomethacin nor α-adrenoceptor blockade attenuated the effect on the release of sympathetic transmitters. The inhibitory action was prevented by pertussis toxin, indicating the involvement of a G protein (Johnson et al. 1991). Furthermore, an inhibitory prejunctional influence of the ANF clearance receptor-binding peptide suggested this receptor played a neuromodulatory role (Johnson et al. 1991). In the guinea-pig vas deferens, the role of ANF seems to be restricted to the purinergic (and not the noradrenergic) transmission component (Mutafova-Yambolieva et al. 1993). No prejunctional effect by ANF on noradrenaline release was observed in guinea-pig atria (Hiwatari et al. 1986) and rat perfused kidney (Schwartz and Eikenburg 1991) in vitro or in dog kidney in vivo (Tomura et al. 1991), indicating possibly a lack of prejunctional functional significance in the organ of origin, the heart, and the major target tissue, the kidney. Experiments with antagonists should clarify whether or not endogenous ANF or related peptides exert a tonic influence on prejunctional sites of the noradrenergic nerves under physiological or pathological conditions. The modulation could then be seen in the context of a mutual feedback system regulating interactions between ANF release and autonomic neurotransmitter release.

2.13 Angiotensin Receptors

Angiotensin II is a hormonal modulator of peripheral sympathetic neurotransmission in vitro and in vivo (Starke 1977; Vanhoutte et al. 1981; Rand et al. 1990; Saxena 1992). Angiotensin I (unless converted into angiotensin II by converting enzyme or less selective proteases) or angiotensin III are often less active or even inactive. Several mechanisms may contribute to an angiotensin II-mediated enhanced organ response to sympathetic nerve stimulation: (i) an increase in the rate of noradrenaline synthesis; (ii) blockade of neuronal noradrenaline reuptake;

(iii) facilitation of noradrenaline release; (iv) an increase in the postjunctional response to noradrenaline. It is widely agreed that iii makes the major contribution to enhanced neurotransmission. Whereas there is no doubt that exogenous angiotensin receptor agonists may facilitate noradrenaline release, there is still much debate over the physiological or pathophysiological role of endogenous angiotensins in activating the prejunctional receptors. Furthermore, it is not clear whether renal angiotensins transported by the blood to other tissues or locally formed hormones represent the primary source of endogenous agonists.

Angiotensin II-induced facilitation appears to work independent of β-adrenoceptor-mediated increase in noradrenaline release in mouse atria and rat tail artery (Rajanayagam et al. 1989). Thus the facilitatory effect of isoprenaline was blocked by propranolol but not by the angiotensin receptor antagonists saralasin or the angiotensin converting enzyme inhibitor captopril excluding a β-adrenoceptor stimulation induced local angiotensin II formation as a final step. On the other hand, the angiotensin II effect was blocked by saralasin. Interestingly, however, in the rat anococcygeus muscle where angiotensin I can be converted into angiotensin II in a captopril-sensitive manner, low saralasin or captopril concentrations failed to affect, but high concentrations blocked the prejunctional isoprenaline effect (Li et al. 1988). In hypertensive humans, however, an association between β-adrenoceptor activation and angiotensin II-mediated facilitation of noradrenaline release on forearm arterioles was suggested (Taddei et al. 1991; see also discussion at the end of this section).

The release of cotransmitters may not be affected by angiotensins in a parallel manner. Thus angiotensin II facilitated evoked tritium (noradrenaline) release upon low and high frequency (2 versus 20 Hz) stimulation, but evoked ATP overflow only at the low frequency (Ellis and Burnstock 1989a). Angiotensin III facilitated tritium overflow also at low and high frequency of stimulation, but inhibited ATP overflow at low and was inactive at high frequency of stimulation. Assuming an exclusive prejunctional source for ATP (which appears unlikely) and disregarding other prejunctional effects mentioned above, the findings would indicate a differential modulation of cotransmitter release by angiotensins.

Angiotensins may initiate events such as increased formation of prostanoids which counteract the prejunctional facilitation. In the guinea-pig vas deferens treated with reserpine (i.e., after noradrenaline depletion, but with the cotransmitter ATP still available), an indomethacin-sensitive inhibition of evoked transmitter release

(determined electrophysiologically) was the predominant effect; in untreated guinea-pig vas deferens, however, a facilitation was observed (Ziogas and Cunnane 1991). In the rabbit vas deferens, angiotensin II facilitated the evoked overflow of [^{3}H]-noradrenaline while angiotensin III inhibited it (Saye et al. 1986). Both angiotensin II and III produced a rise in PGE_2 synthesis. In the presence of indomethacin, both agonists facilitated the release (angiotensin II more than III). This was taken as evidence of interference between a prostanoid-related inhibition (induced predominantly by angiotensin III which is at the same time a comparatively weak AT receptor agonist) and an AT receptor-mediated facilitation. The angiotensin receptor subtype which is responsible for the facilitatory (on noradrenergic response) and inhibitory (on purinergic response) prejunctional effects was selectively blocked by losartan and may belong to the AT_1 type in the rabbit vas deferens (Hegde and Clarke 1993).

Prejunctional angiotensin II receptors appear to interact with other prejunctional receptors. Concomitant activation of inhibitory muscarinic receptors enhanced the facilitatory effect of angiotensin II on evoked tritium (noradrenaline) overflow in the rabbit heart, indicating possibly a prejunctional interaction between the two types of receptors (Garcia-Sevilla et al. 1985). In the canine skeletal muscle in vivo, in which circulating angiotensin II does not influence sympathetic vascular control, inhibition of converting enzyme reduces nerve stimulation-evoked overflow only in the presence of α-adrenoceptor blockade (Schwieler et al. 1991, 1992). It was concluded that inhibitory autoreceptors may override an angiotensin II-mediated facilitation and that local inhibition of angiotensin production or other effects independent of the angiotensin system (e.g. accumulation of bradykinin) may be relevant.

As far as mechanisms involved in prejunctional facilitation are concerned, only limited knowledge is available. In the rabbit isolated iris-ciliary body, facilitation of field stimulation-evoked [^{3}H]-noradrenaline overflow was not enhanced by phosphodiesterase inhibition, excluding an increase in cAMP as a mechanism of facilitation (Jumblatt and Hackmiller 1990). In addition an increase caused by angiotensin II in the overflow of [^{3}H]-noradrenaline evoked by the calcium ionophore A23187 was observed. Therefore, the authors concluded that the angiotensin II effect involved steps beyond calcium influx into the neurone. In the human iris, a saralasin-sensitive prejunctional facilitation of [^{3}H]-noradrenaline overflow was found to be elicited by exogenous angiotensin II (but not by endogenous angiotensin II; Jumblatt et al.

1993). In the cortex of rat kidney, the prejunctional effect of angiotensin II involved a pertussis toxin-sensitive G protein (Murphy and Majewski 1990a) and, in the atria of mice, protein kinase C activation (Musgrave et al. 1991).

In the kidney of anesthetized dogs, locally generated basal levels of endogenous angiotensin II facilitate the evoked noradrenaline release; this is evident from captopril-induced attenuation of the release and the failure of exogenously administered angiotensin II to affect noradrenaline release (Hayashi et al. 1991). Again the receptor responsible was losartan-sensitive and appeared to belong to the angiotensin I type (Suzuki et al. 1992). Reduction of enhanced noradrenaline release by inhibitors of the converting enzyme is reminiscent of findings in pithed rabbits and rats where angiotensin levels are rather high (see Rand et al. 1990 for discussion). These findings, however, cannot necessarily be taken as evidence that endogenous angiotensins have a physiological role in activating the prejunctional receptors. In the anesthetized rabbit with ongoing sympathetic nerve activity, no evidence was found that converting enzyme inhibition had an effect on sympathetic regulation (or that endogenous angiotensin II facilitated cardiovascular sympathetic neurotransmission or enhanced plasma noradrenaline concentration; Szabo et al. 1990). In conscious hypertensive rats, captopril decreased blood pressure, but failed to decrease or even increased the noradrenaline spillover rate into the plasma without affecting the clearance (Kuo and Keeton 1991). These findings do not support the hypothesis that captopril lowers blood pressure in spontaneously hypertensive rats by inhibiting the neuronal release of noradrenaline.

It was assumed that prejunctional angiotensin receptors have a functional role in the state of low sodium intake: In hearts of rats subjected to a low sodium diet (but not in those from rats on a high sodium intake), the stimulation-evoked overflow of endogenous noradrenaline was reduced by inhibition of the angiotensin converting enzyme (Richardt et al. 1991). Under the same condition (low sodium diet) exogenous angiotensin II was inactive, probably because the prejunctional angiotensin II receptors were desensitized by elevated endogenous angiotensin levels. However, in hearts from rats on a high sodium diet, (resulting in a low rate of endogenous angiotensin II formation) exogenous angiotensin I and II reduced the evoked noradrenaline release. It was concluded that low sodium intake increased angiotensin II formation in the heart and resulted in a prejunctional facilitation of noradrenaline release.

The relevance of prejunctional angiotensin effects in humans is still a matter of debate (see Rand et al. 1990; Saxena 1992). No facilitation

by angiotensin was found in humans when reflexly evoked overflow of noradrenaline into the plasma was determined (Seidelin et al. 1987). In functional experiments in which noradrenaline release was not determined, a prejunctional facilitatory effect of angiotensin II on sympathetically mediated arteriolar constriction of human forearm resistance vessels was suggested, since angiotensin II failed to modify the response to exogenous noradrenaline (Seidelin et al. 1991). In hypertensive humans, exogenous angiotensin II infused into the brachial artery enhanced the noradrenaline overflow evoked by the sympathetic activation brought about by applying negative pressure on the lower body, and increased postjunctional vasoconstriction to sympathetic activation but not to exogenous noradrenaline (Taddei et al. 1991). A β-adrenoceptor-mediated facilitation of noradrenaline release induced by the infusion of isoproterenol into the brachial artery was accompanied by enhanced renin and angiotensin II outflow, partly blocked by captopril, and thus apparently at least in part due to formation of angiotensin II and subsequent prejunctional facilitiation. It was not determined by Taddei et al. (1991) whether unstimulated angiotensin II levels imposed a tonic facilitation in hypertensive persons, but the fact that exogenous agonist was active argues against this idea. So far our knowledge about the physiological or pathophysiological role of prejunctional facilitation by angiotensin II is far from complete.

2.14 Neuropeptide Y Receptors

NPY is co-stored in, and co-released with noradrenaline, from sympathetic nerves of various peripheral tissues and functions as a cotransmitter (e.g. Archelos et al. 1987; see Heym and Lang 1986, Campbell 1987, Lundberg et al. 1990a,b, Rand et al. 1990, Kupfermann 1991). As such it could activate prejunctional receptors on sympathetic nerves and thereby inhibit the release of NPY, noradrenaline or other cotransmitters such as ATP (as suggested, for example, for mouse vas deferens by Stjärne et al. 1986). These receptors, however, must be regarded as autoreceptors, at least with regard to the release of NPY, and would thus be beyond the scope of this review. Nevertheless, with regard to the release of noradrenaline, the autoreceptor function in the conventional sense does not apply: Release of NPY from one sympathetic nerve fibre (or even varicosity) could still act at a different sympathetic fibre (or varicosity) and would thus not necessarily act on autoreceptors located on the same varicosity. Therefore, a brief overview of the effects

of exogenous NPY receptor agonists and on receptor classification is given in Table 6. NPY acts presumably by decreasing the influx of calcium ions into the nerve terminal due to selective inhibition of N-type calcium channels (Toth et al. 1993). Like noradrenaline, NPY might participate in neuro-neuronal (transneuronal) interactions and activate heteroreceptors on nerves other than sympathetic ones. Such aspects will be mentioned below in the context of sympathetic–cholinergic interactions (see Warner and Levy 1989, 1990; Ren et al. 1991).

2.15 Sensory Neurotransmitters

The functional significance of sensory neurotransmitters such as tachykinins (e.g. substance P) and calcitonin gene-related peptide (CGRP) was the subject of recent comprehensive reviews (e.g. Holzer 1988, 1992; Maggi 1991; O'Halloran and Bloom 1991; Otsuka and Yoshioka 1993). Compared with the large body of evidence that exists indicating that sensory neuropeptides have an important role as modulators on a postjunctional level, only little information is available on a prejunctional effect on sympathetic nerves. A ganglionic excitation of sympathetic neurotransmission has been reviewed before and will not be discussed here (see Rand et al. 1990). It was suggested that CGRP was a neurotransmitter in nonadrenergic noncholinergic fibres of the cardioaccelerator nerve in guinea-pig atria (Saito et al. 1986). In the rat vas deferens at concentrations less than 2 n*M*, CGRP appeared to have a selective prejunctional inhibitory effect, whereas higher concentrations had non-specific inhibitory postjunctional actions (Ohhashi and Jacobowitz 1985a). In the guinea-pig vas deferens 10 n*M* CGRP reduced the overflow of ATP evoked by stimulation at 2 Hz (but not at 10 Hz), and the twitch response mediated by endogenous ATP; interestingly the evoked overflow of [^{3}H]-noradrenaline remained unchanged under similar conditions (Ellis and Burnstock 1989b). It was shown in this paper that capsaicin, which presumably acts by releasing sensory neurotransmitters, had similar effects, but substance P was inactive. Hence endogenous CGRP might selectively depress the release of ATP from sympathetic nerves without influencing the release of noradrenaline (see also discussion by Maynard et al. 1990). Indirect functional evidence is compatible with the idea of capsaicin mediating release of a sensory peptide (different from substance P or substance K) which inhibits sympathetic neurotransmission prejunctionally but not postjunctionally in the rabbit ear artery (Moritoki et al. 1990). In this tissue, exogenous

Table 6. Modulation of sympathetic neurotransmitter overflow/neurotransmission by NPY receptors

Species	Tissue	Stimulus +parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Rat	Pithed rat	SNS, 1 Hz, 45 s NA	NPY	Inhibition			Inhibition of NA and adrenaline release; inhibition set off by postjunctional facilitatory NPY effects	Dahlöf et al. 1988
Rat	Basilar, superior mesenteric, and femoral artery, femoral and portal vein	FS, 2 or 6 Hz, 2 min ^{3}H	NPY	Inhibition			Despite prejunctional inhibition in mesenteric artery and portal vein (but not in femoral vein) increase in muscular tone	Pernow et al. 1986
Rat	Perfused mesenteric artery bed	FS, 8/10/16 Hz, NA	NPY	Inhibition			Uptake 1 and 2 blocked; at low NPY concentration inhibition, at higher	Westfall et al. 1987
	Perfused mesenteric artery	SNS, 8 Hz NA	NPY, PYY, C-terminal-NPY			Y_2	facilitation of postjunctional responses to NA release. Less NPY inhibition in SHR compared to WKY; probably Y_2 receptor prejunctionally, Y_1 postjunctionally potentiating the response to sympathomimetics	Westfall et al. 1990
Rat	Tail artery	FS, 3 Hz, 6 or 30 p. ^{3}H	NPY	None			Postjunctional potentiation of responses to SNS by NPY, larger effect at short trains, no prejunctional effect observed	Vu et al. 1989

Rat	Portal vein	FS, 2 Hz, 2 min ^3H	NPY	Inhibition		Uptake 1 blocked; inhibition of release but potentiation of postjunctional response	Dahlöf et al. 1985
Rat	Ovary	FS, 10 Hz, 600 p. ^3H	NPY	Inhibition	Y_2	No effect on basal ^3H overflow, no effect of aPP (avian pancreatic polypeptide which is not active at Y_2 receptors)	Ferruz et al. 1992
Rat	Vas deferens	FS, 1 Hz, 300 p. ^3H and ^3H-NA	NPY	Inhibition		Uptake 1 and 2 blocked; inhibition of evoked release and of postjunctional responses to release.	Lundberg and Stjärne 1984
		0.15 Hz, 15 p. Contraction and ^3H	Analogues of NPY and NPY			Effect confined to prostatic part only and insensitive to yohimbine, theophylline, naloxone, picrotoxin or apamin; no effect upon depolarization with high K^+	Donoso et al. 1988
Rat	Anococcygeus muscle	FS, 2–4 Hz, 1 min ^3H	NPY	None		Uptake 1 and 2 blocked; NPY enhanced motor responses by reducing NANC relaxing responses	Vila et al. 1992
Mouse	Atria	FS, 2/5/10 Hz, 1 min ^3H	NPY	Inhibition		With or without phentolamine, inhibition not mediated via decrease of cAMP formation, but reduced by NEM (though not by PTX), not sensitive to PKC inhibitors	Foucart and Majewski 1989 Foucart et al. 1990a Foucart et al. 1990b

Table 6 (*Contd.*)

Species	Tissue	Stimulus +parameter	Agonist	Effect	Antagonist	Subtype	Comment	Reference
Mouse	Vas deferens	FS, 1 Hz, 2 min ^{3}H	NPY PYY	Inhibition			NPY effect independent of blockade of α_2-adrenoceptors	Serfozo et al. 1986
Guinea pig	Heart	SNS, 12 Hz, 1 min NA	NPY	Inhibition			NA uptake 1 blockade enhanced evoked NA overflow but decreased that of NPY-like IR	Haass et al. 1989
Guinea pig	Vas deferens	FS, 5 Hz, trains of 50 p., ^{3}H FS, n.a.p. and e.j.p.	NPY	Inhibition			Less inhibition in immature animals, postjunctional response to FS reduced as well. Inhibition of n.a.p. by NPY not shared by α_2-agonist or ω-conotoxin	Chernaeva and Yankova 1991 Cheung and Dukkipati 1991
Rabbit	Iris ciliary body	FS, 5 Hz, 300 p. ^{3}H	NPY PYY	Inhibition			NPY effect independent of muscarinic receptor and α_2-adrenoceptor blockade	Ohia and Jumblatt 1990b
Rabbit	Ear artery	FS, 1 Hz, 30 s ^{3}H	NPY	Inhibition			Inhibition of evoked overflow slowly reversible; postjunctional facilitation of response	Wong-Dusting and Rand 1988; Rand et al. 1990
Dog	Gracilis muscle	FS, 2/10/ 20 Hz, 2 min NA	NPY	Inhibition			Co-release of NA and NPY-like IR determined; NPY preferentially released by high frequency stimulation	Pernow et al. 1988

Pig	Spleen in vivo	SNS, 2/10/ 20 Hz, 240 p. NA and ^{3}H-NA	NPY	Inhibition	NPY induced inhibition of release, but strong postjunctional vasoconstriction	Lundberg et al. 1989
Pig	Choroid plexus	FS, 5 Hz, trains or bursts of 25 Hz ^{3}H	NPY	Inhibition	Desipramine, bacitracin, phenylmethylsulfonyl fluoride present	Nilsson et al. 1990
Pig	Kidney in vivo	SNS, 5 Hz, 1 min NA	PYY	Inhibition	Release of both, NA and cotransmitter NPY, inhibited	Pernow and Lundberg 1989
Man	Iris ciliary body	FS, 5 Hz, 300 p. ^{3}H	NPY	Inhibition	Uptake 1 blocked	Jumblatt et al. 1993
Man	Mesenteric vein	FS, 6 Hz, 30 s ^{3}H	NPY	Inhibition	NPY induced inhibition nifedipine insensitive; postjunctional responses in many more human vessels investigated	Pernow et al. 1987
Man	Forearm blood flow	Reflex SNS	NPY	None	Vasoconstriction by NPY, but no modulation in vivo of effects of endogenous or exogenous NA	Clarke et al. 1991
Man	Submandibular arteries	FS, 6 Hz, 2 min ^{3}H	NPY	Inhibition	Inhibition of release, but potentiation of postjunctional response to NA release	Lundberg et al. 1985

For explanations and codes, see Table 1.
NPY, neuropeptide Y; PYY, peptide YY; IR, immunoreactivity; NEM, N-ethylmaleimide; PKC, protein kinase C; PTX, pertussis toxin; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; n.a.p., nerve action potential; e.j.p., excitatory junction potential.

CGRP inhibits responses to field stimulation and to exogenous noradrenaline to a similar extent (Maynard et al. 1990). Substance P inhibited the evoked release of noradrenaline in the perfused mesenteric vasculature of normotensive rats and less so in spontaneously hypertensive rats (Tsuda et al. 1988). In contrast, in the mesenteric vascular bed of normotensive rats, prejunctional modulation of noradrenaline release was found neither for capsaicin-induced release of endogenous sensory peptides nor for CGRP, substance P, or neurokinin A or B (Kawasaki et al. 1990). Nevertheless, a dense sensory innervation and strong postjunctional effects of sensory neurotransmitter depletion were observed. No effect was seen with substance P when overflow of noradrenaline was determined in the perfused gracilis muscle in dogs (Kahan et al. 1985) or when functional responses to noradrenaline release of guinea-pig trachea were taken as prejunctional parameter in the presence of substance P and neurokinin A (Pendry and Maclagan 1991b). It has been suggested that a sympathetic cotransmitter function of an endogenous sensory neuropeptide (acting via an NK_2 receptor) was responsible for indirect facilitation induced by nicotine of cholinergic neurotransmission in guinea-pig bladder (Shinkai et al. 1991), and this will be discussed below.

2.16 Opioid Receptors

Opioid receptor modulation of sympathetic neurotransmission has been the subject of recent reviews (Starke 1977; Vizi 1979; Fuder 1988; Illes 1989). Opioids are stored in (Wilson et al. 1980; Lang et al. 1983; North and Egan 1983; Reinecke and Forssmann 1984; Weihe et al. 1985; Douglas et al. 1986; De Potter et al. 1987; Krukoff 1987) and may be released from peripheral sympathetic nerves of organs in different species (Lang et al. 1983; Xiang et al. 1984; De Potter et al. 1987). Since 1989 (the time of the last comprehensive review on prejunctional opioid mechanisms), comparatively little more direct evidence has been accumulated for a functional role of opioid receptor-mediated modulation of noradrenaline release. To our knowledge, no physiological role of endogenous opioids in regulation of peripheral sympathetic neurotransmission has been demonstrated so far. An interesting interaction between noradrenergic and cholinergic neurotransmission was observed with respect to opioid receptor-mediated effects (Nakayama et al. 1990): Whereas inhibitory μ-opioid receptors were present on cholinergic and noradrenergic nerves in the

guinea-pig ileum, inhibitory κ-receptors were functionally relevant only on cholinergic neurones. Further, the μ-agonist [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]enkephalin (DAMGO) showed a preferential high potency for receptors on noradrenergic nerves. Surprisingly, low concentrations of the μ-agonist facilitated the evoked acetylcholine release. This facilitation was not seen in the presence of yohimbine. The findings suggest that a preferential inhibition of noradrenergic tone by activation of μ-receptors reduced the α_2-adrenergic inhibition of the tone of cholinergic nerves in the ileum (Nakayama et al. 1990).

Nevertheless, exogenous opioids have been shown to inhibit noradrenaline release in numerous tissues in vitro and in the intact animal in vivo. The effects are mediated by a variety of subtypes of opioid receptors which may differ from tissue to tissue in the same species, or within the same tissue in different species (see Illes 1989). As a rule, and like prejunctional modulation by many other receptors, opioid receptor-mediated inhibition appears to be inversely related to stimulation intensity, as described by frequency and train length (see Duckles and Budai 1990). In the rat vas deferens, purinergic and noradrenergic transmission was inhibited in a similar manner by opioid μ-, δ-, and κ-agonists, indicating that release of cotransmitters may underly a parallel regulation mechanism (Driessen et al. 1993). Like other prejunctional receptors, opioid receptors interact with other prejunctional receptors, for example κ-receptors in rabbit tissues (Limberger 1988) or μ-receptors with adenosine A_1 receptors or α_2-adrenoceptors in rat blood vessels (Bucher et al. 1992b; see also Illes 1989). No indication of an opioid receptor reserve was found (κ-subtype exclusively present) on sympathetic nerves of the guinea-pig atria (Fuder 1988).

2.17 Other Neuroactive Polypeptides

In addition to the peptides discussed above, a variety of other polypeptides modulate the release of noradrenaline in peripheral tissues. In the vas deferens of mouse and rat, a calcium-dependent facilitation of tritium (noradrenaline) release evoked by field stimulation (mediated by bradykinin B_2 receptors probably) was observed in the presence of high bradykinin concentrations (Llona et al. 1991). This is a surprising finding, since an inhibitory effect was previously seen with low bradykinin concentrations in rabbit tissues (Starke et al. 1977). As discussed in Sect. 2.9, the latter effect involved release of prostanoids which might not be generated to an sufficient extent in all tissues.

A second example of a facilitatory prejunctional effect is that induced by adrenocorticotropic hormone (ACTH). Thus evoked release of noradrenaline in the pulmonary artery (Göthert 1984; Costa and Majewski 1988), aorta (Göthert 1984), atrial strips (Costa and Majewski 1988) and perfused heart (Szabo et al. 1988) of the rabbit was enhanced by low ACTH concentrations. The effect was independent of neuronal reuptake and β-adrenoceptor blockade. In contrast, no ACTH effect was seen in rat atria, rat pulmonary artery and guinea-pig pulmonary artery (Costa and Majewski 1988), indicating a species difference between rabbit and rat or guinea pig. It was suggested that cAMP might be involved in the mechanism of ACTH-mediated facilitation in rabbit pulmonary artery (Göthert and Hentrich 1984). In the pithed rabbit, facilitation of sympathetic neurotransmission was reflected in an increase in the noradrenaline spillover rate (Szabo et al. 1987), an effect which was independent of β-adrenoceptor blockade and did not involve adrenal steroids. Whereas a physiological function was considered unlikely by the authors, a pathophysiological role was not excluded.

As far as vasoactive intestinal polypeptide (VIP) is concerned, inconsistent results were observed. No prejunctional effect was seen in guinea-pig trachea (Pendry and Maclagan 1991b) and dog gracilis muscle (Kahan et al. 1985), but facilitation was observed in the presence of VIP (and of peptide histidine isoleucine) in the choroid plexus of pigs (determined with [^{3}H]-noradrenaline release, Nilsson et al. 1990) and in chick sympathetic neurones in culture (Przywara et al. 1991). The facilitation in chick neurones was not accompanied by modification of intraneuronal concentrations of calcium ions. It was suggested that galanin, another neuroactive peptide which was shown to inhibit noradrenaline release in rat medulla oblongata (Tsuda et al. 1992) and to be co-stored with dopamine β-hydroxylase in subpopulations of rat renal and splenic nerves (Longley and Weaver 1993) facilitated sympathetic neurotransmitter release in the rat vas deferens (Ohhashi and Jacobowitz 1985b), although only indirect evidence was presented by facilitation of the postjunctional response to field stimulation (whereas the effect of exogenous noradrenaline was unchanged by low galanin concentrations). The significance of these findings is unclear.

Anecdotal reports exist on inhibitory prejunctional effects of a few other polypeptides. For instance, somatostatin inhibited the release of [^{3}H]-noradrenaline evoked by field stimulation in rabbit ear artery (Maynard et al. 1991), and indirect evidence (reduction of postjunctional response to field stimulation by somatostatin) was previously presented for the rat vas deferens (Magnan et al. 1979).

Somatostatin-induced inhibition (like [Met^5]enkephalin and noradrenaline) appears to decrease a voltage-dependent calcium current and to increase a potassium conductance, both events involving a pertussis toxin-sensitive G protein (Surprenant et al. 1990). Neurotensin inhibited the evoked release of noradrenaline to a larger extent in normotensive than in spontaneously hypertensive rats (Tsuda et al. 1988), indicating possibly a pathologically relevant prejunctional influence. A decrease by insulin of evoked overflow of endogenous noradrenaline from rat isolated mesenteric arteries was cocaine sensitive (Shimosawa et al. 1992), and hence probably not due to true prejunctional inhibition but to an insulin-dependent increase in neuronal noradrenaline reuptake which may distort the ratio between the neurotransmitter appearing in the overflow and that actually released.

3 Heteroreceptors Modulating Acetylcholine Release

3.1 Localization of Prejunctional Receptors

In most isolated organs used for studies of prejunctional mechanisms, the adrenergic nerves are postganglionic, and hence the point of attack of drugs is the terminal or preterminal portion of the sympathetic neurone. A completely different situation prevails in the case of cholinergically innervated organs: With only a few exceptions – circular muscle preparation of guinea-pig ileum (Kilbinger et al. 1992a) and pig iris (Schaeppi 1966) – the preparations used contain cholinergic ganglia (airways, heart, gastrointestinal and urogenital tracts). Consequently, any modulatory effect on acetylcholine release observed and usually designated as "prejunctional" can derive from an action on (a) presynaptic terminals of a preganglionic nerve in a ganglion, (b) the ganglion cell proper (and then being postjunctional), and (c) the relatively short preterminal and/or the terminal region of the postganglionic part of the neurone. This has already been stated in the seminal publication by Paton and Vizi (1969).

In the experiments to be reported in the present chapter, a combination of points (a) to (c) cannot safely be excluded, but two kinds of observation favour the view that measurement of acetylcholine release evoked by field stimulation is a major index of (c), namely:

1. When orthodromic parasympathetic nerve stimulation is carried out in the presence of ganglionic blocking agents, acetylcholine release

is greatly depressed – by 88% in the perfused chicken heart according to Löffelholz (1981); by 90% according to Muscholl and Muth (1982) or 96% according to Habermeier-Muth and Muscholl (1988) in the rabbit perfused atria; by 80% in the rat perfused heart according to Bognar et al. (1990b); and by 84% in the rat isolated trachea according to Wessler et al. (1991) – but electrical field stimulation of heart preparations (Löffelholz 1981) or the trachea (Wessler et al. 1991) evokes hexamethonium- and (+)-tubocurarine-resistant acetylcholine release. Likewise, in the guinea-pig myenteric plexus, acetylcholine release evoked by field stimulation is unaffected by ganglionic blocking agents (Kilbinger 1988).

2. In the myenteric plexus, acetylcholine release evoked by a high K^+ concentration (both in the presence or absence of tetrodotoxin), which precludes impulse traffic along the axons, is modulated by oxotremorine (decrease) or atropine (increase), although to a lesser extent than release after electrical field stimulation (for discussion and references, see Kilbinger 1988). Thus, major agonist-induced alterations in the release of acetylcholine evoked by high K^+ or field stimulation do not exclude alterations in the excitability of the ganglion cell but, nevertheless, they indicate a functional consequence of receptor activation on the terminals.

Admittedly, in vivo terminal nerve fibres are activated through invasion of impulses carried along the preganglionic path. In other words, using the above methodology, the location of the receptor-mediated event can be pinpointed but the physiological relevance has to be ascertained by other means. By contrast, orthodromic stimulation of the preganglionic nerves, as used in many in vivo or in some in vitro experiments on airways, heart or urogenital tract preparations, utilizes the physiological pathway (although not necessarily in vivo stimulation modes), but does not allow to exclude ganglionic transmission as a possible point of attack of the agonist or antagonist under study. However, in order to test the physiological relevance of an assumed autacoid-mediated mechanism, or its validity to explain pharmacological effects occurring in vivo, the latter methodology is indispensable.

Finally, even under the rigorous conditions of prevailing postganglionic activation of acetylcholine release, clarifying experiments as described above for muscarinic autoreceptors have not generally been performed with the majority of agonists listed in the tables and claimed to produce heteroreceptor-mediated *prejunctional* effects. An exception is the inhibitory α_1-adrenoceptor on cholinergic nerves of the rat heart which can be demonstrated for release evoked by high K^+ (Wetzel and

Brown 1985; Wetzel et al. 1985; Benkirane et al. 1986; McDonough et al. 1986), electrical field (Wetzel and Brown 1985) or vagus nerve stimulation (Bognar et al. 1990a). Likewise, the facilitatory α_1-adrenoceptor-mediated response in the rat heart was observed both after vagus nerve and electrical field stimulation (Bognar et al. 1990a). However, for the majority of papers refereed in the tables, a word of caution has to be added as far as the receptor location is concerned, unless there is independent corroborative evidence from studies confined to those individual parts of the neuronal pathway where signal transfer might be impeded.

3.2 Adrenoceptors

The original observations of an adrenoceptor-mediated control of acetylcholine release were carried out on intestinal preparations of different species (Paton and Vizi 1969; Beani et al. 1969; Kosterlitz et al. 1970; Del Tacca et al. 1970; Vizi and Knoll 1971). The rich cholinergic innervation was an advantage for the biological acetylcholine assay commonly employed at that time. These and several subsequent papers (for review of earlier work, see Vizi 1979; Wikberg 1979) showed that adrenaline, noradrenaline or sympathetic nerve stimulation decrease acetylcholine overflow provided the stimulation rate is low, or cholinergic nerve activity is maintained spontaneously (see Table 7). At stimulation frequencies of 10 Hz or higher, adrenergic modulation is difficult to obtain but can be demonstrated when the number of pulses applied is kept low (e.g. Wessler et al. 1987). Such findings and the observations of an inverse relationship between α-adrenoceptor agonist potency to inhibit acetylcholine release and external calcium concentration (reviewed by Illes 1986) support the idea of presynaptic α-adrenoceptors regulating calcium entry into the neurone during excitation. However, restriction of calcium entry into the neurone might be secondary to a hyperpolarizing action caused by increase in potassium conductance mediated by an α-adrenoceptor; there is indirect evidence for this as well as for the calcium hypothesis (Illes 1986; North 1986). Either of the ionic changes could explain hindrance of impulse propagation into the terminals by presynaptic receptors as the functionally relevant mechanism of action in prejunctional inhibition (Stjärne 1989) as opposed to local regulation of excitation-secretion coupling at the site of transmitter release, but none of the possibilities mentioned mutually excludes the others. Thus, despite great experimental efforts these questions are still undecided,

Table 7. Modulation of cholinergic neurotransmitter overflow/neurotransmission by α-adrenoceptors

Species	Tissue	Stimulation +parameter tested	Agonist	Effect on ACh release	Antagonist	Subtype	Comment	Reference
Chicken	Heart (perfused)	VNS, 5 Hz, 30 s ^{3}H overflow	Methoxamine, clonidine	None			No evidence for prejunctional α_1- or α_2-receptor modulating ACh outflow	Löffelholz et al. 1984
Rat	Atria	K^+ 57 m*M*, 2.9 min	(−)-NA, (+)-NA, adrenaline	Inhibition	Yohimbine, propranolol	α	(−)-NA and adrenaline decreased release; NA was antagonized by yohimbine 10 μM but not by propranolol. (+)-NA without effect. After FS similar decrease as after K^+-evoked release	Wetzel and Brown 1985
		FS, 5 Hz, 2.9 min ^{3}H (ACh) overflow	NA	Inhibition		(α)		
Rat	Atria	K^+ 57 m*M*, 2.9 min	NA, adrenaline, methoxamine	Inhibition	Prazosin, yohimbine	α_1	No modulation by α_2- or β-receptor; prazosin K_i = 0.4 n*M*, yohimbine K_i= 40 n*M* against NA	Wetzel et al. 1985
		^{3}H (ACh) overflow	Clonidine, isoprenaline	None				
Rat	Atria	K^+ 57 m*M*, 3 min	NA, α-methyl-NA, phenylephrine	Inhibition	WB4101, Wy26703, rauwolscine and other	α_1	Decrease by NA only blocked by α_1- but not by α_2-antagonists. K_A for NA after phenoxybenzamine = 13 μ*M* as for other α_1-receptors. Little or no receptor reserve	McDonough et al. 1986
		^{3}H (ACh) overflow	BHT920, UK14304	Small inhibition None				
			Amidephrine, ST587, cirazoline	None			Efficacy too low. Action as antagonists against NA	

Rat	Atria	K^+ 100 m*M*, 2 min	NA, methoxamine	Inhibition	Prazosin, idazoxan, propranolol, sulpiride	α_1	Decrease by NA only blocked by α_1-antagonist; α_2-agonist ineffective: site of action either prejunctional or somato-dendritic α_1-receptor. NA released but inhibitory α_1-receptor not stimulated	Benkirane et al. 1986
			UK14304	None				
		^3H overflow	Amphetamine, β-phenethylamine tyramine	None				
Rat	Heart (perfused)	VNS, 3 Hz, 6 min, or 10 Hz, 2 min	NA	Inhibition		α_1	Decrease of ACh overflow in the presence of yohimbine and propranolol. Both imidazolines enhanced ACh overflow. Increase after oxymetazoline blocked by α_1- but not by α_2-antagonists	Bognar et al. 1990a
			Oxymetazoline, xylometazoline	Facilitation	Phentolamine, prazosin, (−)-WB4101, SKF104078, rauwolscine, idazoxan, yohimbine	α_1		
		^{14}C-ACh overflow Heart rate atrial force					No enhancement of postjunctional effects of VNS	
Rat	Heart in vivo	VNS, 4–5 Hz, 2.5 min	Phenylephrine, BHT920	Inhibition None	Prazosin yohimbine, rauwolscine	α_1	Attenuation of vagally induced bradycardia by α_1- but not α_2-agonist. Effect of phenylephrine antagonized by α_1- but not α_2-blockade	McGrattan et al. 1987
		Heart rate						
Rat (pithed)	Heart in vivo	Drug injection	Phenylephrine, amidephrine	Increase Increase	Prazosin, WB4101, corynanthine	α_1	Negative chronotropic effect of α_1-agonists was enhanced by neostigmine and blocked by atropine, TTX or hexamethonium, as well as by α_1-antagonists. Conclusion: α_1-mediated release of ACh occurs at level of parasympathetic ganglia	Flavahan and McGrath 1982
		Heart rate	Xylazine	None				

Table 7 (*Contd.*)

Species	Tissue	Stimulation +parameter tested	Agonist	Effect on ACh release	Antagonist	Subtype	Comment	Reference
Guinea pig	Trachea	FS, 20 Hz, 1 s Contraction	NA, adrenaline	Inhibition	Prazosin Yohimbine, corynanthine, rauwolscine	α_2	In the presence of propranolol NA or adrenaline inhibited FS evoked but not ACh evoked contraction. Inhibitory effect selectively antagonized by α_2-AR blockers	Grundström et al. 1981b
Guinea pig	Superior cervical ganglion	FS, 1–8 Hz, 10 min ^{3}H overflow	NA	Inhibition	Phentolamine, prazosin, yohimbine, propranolol	α_2	Decrease in ACh overflow blocked by α_2- but not by α_1- or β-antagonists; inhibition inversely correlated with stimulation rate.	Belluzzi et al. 1987
			Isoprenaline	Facilitation		β	Increase by isoprenaline in the presence of yohimbine	
Guinea pig	Atria	FS, 2 Hz, 30 s ^{3}H overflow	NA	Inhibition	Idazoxan, phentolamine, prazosin	α_2	Decrease by NA blocked by α_2- but not by α_1-antagonist. Intrinsic activity of clonidine too low.	Loiacono and Story 1986
			Clonidine	None				
		VNS, 2–16 Hz, 10–30 s rate of beat	Endogenous NA	None	Above drugs		No endogenous NA tone as shown by failure of antagonists to enhance ACh overflow, also in the presence of cocaine. VNS evoked decrease in rate of beat unaffected by NA in the presence of propranolol	
			NA	None				
Guinea pig	Right atrium	FS, 2 Hz, 2 min	NA clonidine	None	Prazosin, CH38083		Neither α_1- nor α_2-receptor stimulation altered overflow.	Manabe et al. 1991

		^{3}H overflow	Phenylephrine				Endogenous NA tone ruled out by ineffectiveness of α_1- or α_2-antagonist to enhance overflow	
Guinea pig	Heart	FS, 20 Hz, 2 min ACh (B) overflow	Endogenous NA	None			ACh overflow evoked by FS unaltered versus controls after pretreatment of animals with reserpine which abolished NA release	Dieterich et al. 1978
Guinea pig	Ileum	FS, 0.3–10 Hz ACh (B) overflow	NA, adrenaline, methoxamine, isoprenaline, DA	Inhibition None	Phenoxybenzamine, phentolamine, ergotamine	α	Decrease of basal and evoked (0.3–0.7 Hz) ACh overflow antagonized by the α-blockers indicated; no significant inhibitory effects at FS of 1–10 Hz. After pretreatment with reserpine basal and evoked ACh overflow (0.3–3 Hz) were enhanced versus controls: endogenous NA tone	Paton and Vizi 1969
Guinea pig	Ileum	FS, 0.3 Hz, ACh (B) overflow	NA, adrenaline, isoprenaline	Inhibition Inhibition None	Phenoxybenzamine, propranolol	α	Basal and evoked ACh overflow decreased only by NA and adrenaline, effect antagonized by phenoxybenzamine but not by propranolol .	Kosterlitz et al. 1970
		Contraction	see above	Inhibition	see above		Inhibitory effects of all catecholamines on contraction caused by exogenous ACh unaffected by phenoxybenzamine but antagonized by propranolol: postjunctional β-receptors present	

Table 7 (*Contd.*)

Species	Tissue	Stimulation +parameter tested	Agonist	Effect on ACh release	Antagonist	Subtype	Comment	Reference
Guinea pig	Ileum	FS, 0.3–10 Hz, 3 min ACh (C) overflow	Endogenous NA	Inhibition	Yohimbine, tolazoline, phentolamine	α	At 0.3 and 3 Hz but not at 10 Hz enhanced ACh overflow after yohimbine suggesting endogenous inhibitory noradrenergic tone	Kilbinger and Wessler 1979
Guinea pig	Ileum	FS Contraction	Clonidine, tramazoline oxymetazoline, NA, xylazine, phenylephrine, methoxamine	Inhibition (order of potency of drugs in list decreasing)	Tolazoline, phentolamine, phenoxy-benzamine	α_2	Order of potency of series of agonists to inhibit FS evoked contraction supporting idea that α_2-receptor mediates inhibition of ACh release	Wikberg 1979
Guinea pig	Ileum	FS, 0.5 Hz, 5 min ^{3}H overflow	NA	Inhibition	Yohimbine	α_2	Effect of ACh antagonized by yohimbine with apparent K_D similar to that for α_2-receptor of adrenergic nerves	Alberts and Stjärne 1982
Guinea pig	Ileum	FS, 0.13 Hz, 5 min ACh (B) overflow, contraction	NA	Inhibition	Phentolamine, prazosin, yohimbine, phenoxy-benzamine	α_2	Decrease of basal and evoked ACh overflow as well as inhibition of evoked twitches of the ileum blocked by phentolamine or yohimbine but not by prazosin or phenoxybenzamine	Fagbemi and Salako 1982
Guinea pig	Ileum	FS, 0.1 Hz ^{3}H (ACh) overflow	Endogenous NA	Inhibition		AR	Increase of evoked ACh overflow from ileal strips of reserpine-treated animals	Kilbinger and Pfeuffer-Friederich 1985
Guinea pig	Ileum	Substance P evoked	NA	Inhibition	Yohimbine	α_2	TTX-sensitive decrease of substance P evoked ACh	Vizi and Bartho 1985

		transmitter release ^{3}H overflow					overflow antagonized by yohimbine, suggesting excitatory effect of peptide on ganglionic cell body	
Guinea pig	Ileum	FS, 2 Hz, 15 min ACh (C) overflow	NA	Inhibition		AR	For dog airways see below	Martin and Collier 1986
Guinea pig	Ileum	FS, 0.1–10 Hz, 3.3–0.2 min ^{3}H overflow	NA	Inhibition	Tolazoline	α_2	Concentration-response curves of NA similar at 0.1, 1 and 10 Hz. Evoked ACh overflow not inhibited by NA in the presence of physostigmine indicating inefficiency of α_2-modulation when autoreceptor is strongly activated	Wessler et al. 1987
Guinea pig	Ileum submucosal neurones	Focal electrical stimulation; cell membrane potential; nicotinic e.p.s.p.; sympathetic nerve-evoked vasoconstriction	UK14304 oxymetazoline	Membrane hyperpolarization (postjunctional); inhibition of e.p.s.p. (prejunctional); inhibition of vasoconstriction (prejunctional)	Idazoxan, yohimbine, SKF104078, WB4101, ARC239	α_{2A}	Antagonists exhibited similar apparent dissociation constants for blocking post- and prejunctional effects to UK 14304. Neither prazosin nor chlorpromazine altered responses to UK14304. Oxymetazoline was partial agonist on pre- and postjunctional cholinoceptor	Shen et al. 1990
Guinea pig	Ileum	FS, 1 Hz, 3 min	Xylazine, oxymetazoline, detomidine,	Inhibition	WB4101, CH38083, idazoxan,	α_{2A}	Agonist-induced decrease in ACh overflow antagonized by idazoxan, WB4101 and CH	Blandizzi et al. 1991, 1993

Table 7 (*Contd.*)

Species	Tissue	Stimulation +parameter tested	Agonist	Effect on ACh release	Antagonist	Subtype	Comment	Reference
		^{3}H overflow	α-Methyl-NA	Inhibition	prazosin, ARC239		38083 but not by prazosin or ARC239	
Guinea pig	Colon	VNS, 1 Hz, FS, 1 Hz, 5 min	NA, adrenaline	Inhibition		AR	Spontaneous or evoked (VNS; FS) ACh overflow decreased. At 10 Hz VNS or FS no inhibitory effect of amines. Evoked (VNS, FS) release inhibited by extensive (50 Hz, 10 min) SNS	Beani et al. 1969
		ACh (B) overflow	Endogenous NA released by SNS	Inhibition		AR		
Guinea pig	Colon	FS, 4 Hz, 10 min	NA, DA, clonidine	Inhibition	Yohimbine	α_2	Potency order for decrease of basal overflow: clonidine > DA = NA; for evoked overflow: clonidine > NA > DA; all agonist effects blocked by yohimbine.	Marcoli et al. 1985
		FS, 1 Hz, SNS, 40 Hz, ACh (B) overflow	Endogenous NA	Inhibition	Yohimbine	α_2	Inhibition by SNS of FS-evoked ACh overflow and peristaltic reflex, both antagonized by yohimbine	
Rabbit	Superior cervical ganglion	Preganglionic NS, 0.3–10 Hz ACh (B) overflow	NA, adrenaline	Inhibition	Phentolamine	α	Decrease of ACh overflow evoked by 0.3 Hz stimulation antagonized by phentolamine. No agonist effect on basal or 10 Hz-stimulated overflow	Dawes and Vizi 1973
Rabbit	Atria (perfused)	VNS, 2 Hz, 3 min	Endogenous NA released by SNS 2 Hz	Inhibition	Prazosin, idazoxan, rauwolscine	α_1	When SNS pulses were applied 19 ms before VNS pulses ACh overflow was increased.	Muscholl and Habermeier-Muth 1991a

		^{14}C-ACh overflow	(constant pulse-to-pulse interval)		propranolol		Further enhancement after prazosin but not after α_2- or β-antagonists.	
		NA (C) overflow	ibid.	Facilitation		Non-adrenergic	Increase in evoked ACh overflow by SNS not affected by pretreatment with reserpine but abolished by guanethidine: non-adrenergic facilitatory co-transmitter likely.	Muscholl and Habermeier-Muth 1991b
		VNS, 2 Hz, 3 min see above	Oxymetazoline	Facilitation	Prazosin	α_1?	Oxymetazoline at 2.5 μM but not at 20–500 nM enhanced overflow. Blocked by prazosin 20 nM	
Rabbit	Jejunum	FS, 0.5 Hz, 20 min	NA	Inhibition		AR	Decrease of basal or evoked (0.5 Hz) overflow.	Vizi and Knoll 1971
		ACh (B) overflow	Endogenous NA released by SNS 20 Hz	Inhibition		AR	Decrease of basal ACh overflow; not seen after pretreatment with reserpine	
Rabbit	Jejunum	FS, 0.4 Hz, 5 min	Endogenous NA released by SNS 10 Hz	Inhibition		AR	Evoked ACh overflow decreased by SNS which failed to inhibit ACh release after pretreatment of rabbits with 6-OH-DA	Manber and Gershon 1979
Cat	Superior cervical ganglion	Preganglionic NS, 1–20 Hz	NA clonidine	Inhibition	Yohimbine, phentolamine, prazosin	α_2	Agonists decreased ACh overflow evoked by 20 Hz but not 1–10 Hz NS; no effect on basal outflow. Potency of antagonists to block inhibition suggests α_2-receptor. At 20 but not at 5 Hz NS yohimbine or phentolamine enhanced ACh overflow.	Araujo and Collier 1986
		ibid. 20 Hz	Endogenous NA	Inhibition				
		ACh (C)						

Table 7 (*Contd.*)

Species	Tissue	Stimulation +parameter tested	Agonist	Effect on ACh release	Antagonist	Subtype	Comment	Reference
		overflow ^{45}Ca uptake	NA	Inhibition	Yohimbine, phentolamine	α_2	Inverse relationship between Ca^{2+}concentration and % inhibition by NA of ACh overflow; ^{45}Ca accumulation in ganglia also inhibited by NA via α_2-receptor	Araujo and Collier 1987
Dog	Trachea	FS, 2 or 10 Hz, 5×1 min,	NA	None	Endogenous NA release prevented by guanethidine		No modulation of ACh release by exogenous or endogenous NA acting on α-AR (but see above, guinea-pig ileum)	Martin and Collier 1986
	Bronchi	ibid., 2 Hz ACh (C) overflow	NA	None				
Dog	Submaxillary gland	Chorda tympani NS, 10 Hz, 10 s Kallikrein release	Endogenous NA	Inhibition	Yohimbine	α_2	NS-evoked and basal kallikrein release into saliva as well as salivary flow increased by yohimbine suggesting prejunctional α_2-receptor	Girolami et al. 1991
Man	Taenia coli	FS, 2 Hz ACh (B) overflow	NA Isoprenaline	Inhibition None		α	Evoked but not basal ACh overflow decreased by NA but not by isoprenaline	Del Tacca et al. 1970

For chemical designation of code names, see the references cited.
FS, electrical field stimulation; NS, nerve stimulation; VNS, vagus nerve stimulation; SNS, sympathetic nerve stimulation; AR, adrenoceptor; TTX, tetrodotoxin; ^3H overflow, total activity determined in superfusate after loading tissue with [^3H]-choline; ^3H-ACh, [^3H]-ACh (+ [^3H]-choline derived from [^3H]-ACh) separated from other [^3H] compounds routinely; ^3H (ACh), routinely [^3H] determinations but in selected experiments fractionation of [^3H] compounds; ACh (B), ACh determined by bioassay, presence of cholinesterase inhibitor; ACh (C), ACh determined chemically, presence of cholinesterase inhibitor.

both for hetero- and autoreceptor-mediated modulatory processes (Starke et al. 1989).

As borne out by Table 7, inhibitory modulation of acetylcholine release by an α_2-adrenoceptor is generally observed on intestinal preparations of different species, and earlier observations where receptor classification was not possible due to lack of selective drugs do not contradict specified classifications obtained later on. Similarly, the acetylcholine release from superior cervical ganglia is reduced by α_2-adrenoceptor activation, either by exogenous agonists or endogenously released noradrenaline. In the rat heart, inhibition of acetylcholine release is mediated by an α_1-adrenoceptor which is also valid for the rabbit atrium. However, in the chicken heart prejunctional control of acetylcholine release by adrenoceptors is absent, and for guinea-pig atria there are controversial findings. Loiacono and Story (1986) reported an inhibition of field stimulation-evoked acetylcholine release after exogenous noradrenaline that was mediated by an α_2-adrenoceptor, but failed to notice an endogenous noradrenergic tone unmasked by adrenoceptor antagonists even in the presence of cocaine (expected to raise the biophase concentration of noradrenaline). Also using 2 Hz field stimulation of guinea-pig atria, Manabe et al. (1991) confirmed the absence of modulatory effects of endogenous noradrenaline but did not observe any inhibitory effect of exogenous noradrenaline on acetylcholine release. As discussed by Loiacono and Story (1986), the prejunctional α-adrenoceptors on cholinergic nerves might be remote from the sites of noradrenaline release, possibly on preganglionic cholinergic nerves. However, this would not explain the lack of effect of exogenous noradrenaline on acetylcholine release reported by Manabe et al. (1991). Another point which requires further studies is the observation on guinea-pig atria that chronotropic responses to extrinsic vagus nerve stimulation 2–16 Hz were unaffected by noradrenaline in the presence of propranolol (Loiacono and Story 1986), whereas acetylcholine overflow evoked by field stimulation at 2 Hz was decreased (see above). In contrast, mechanical responses of guinea-pig ileum are fairly well correlated to prejunctional inhibitory effects on evoked acetylcholine release (Fagbemi and Salako 1982). In addition to the inhibitory α_1-adrenoceptor in rat and rabbit heart (see above) there is recent evidence for an excitatory α_1-adrenoceptor facilitating evoked acetylcholine release in these preparations (Bognar et al. 1990a; Muscholl and Habermeier-Muth 1991b).

Only recently, acetylcholine overflow has been measured in airway preparations. In rat trachea the inhibition of release is caused indirectly

by β_1-adrenoceptor-mediated liberation from airway epithelium of a prostanoid acting transsynaptically (Reinheimer et al. 1993) or, using the terminology proposed by Rand et al. (1990), transjunctionally. However, for feline, canine and human trachea or bronchi, there is only indirect evidence which supports a β-adrenoceptor-mediated prejunctional inhibition of cholinergic transmission, in most cases classified as a β_2-adrenoceptor (Table 8). Using selective β_1- and β_2-adrenoceptor antagonists and studying both contractions and excitatory junction potentials (EJPs) of canine bronchi after 1–10 Hz field stimulation, Janssen and Daniel (1990) obtained evidence of both β_1 and β_2 prejunctional receptors inhibiting cholinergic neurotransmission. Close appositions of adrenergic and cholinergic nerve varicosities were seen more frequently in canine bronchi than in trachea, a finding which is consistent with an earlier observation of adrenergic prejunctional control of EJPs lacking in dog trachea. However, neither the stimulation-evoked release of endogenous acetylcholine from dog trachea nor that from dog bronchi was found to be modulated by either α- or β-adrenoceptor stimulation through endogenous or exogenous noradrenaline (Martin and Collier 1986; Tables 7, 8). Different intensities of electrical field stimulation might be one reason for the apparent discrepancy between the two last-mentioned studies regarding prejunctional control of acetylcholine release (Janssen and Daniel 1990); another reason is the presence of both physostigmine and atropine in the experiments with endogenous acetylcholine determinations, as opposed to the functional studies in which postjunctional effects are measured as parameter of the acetylcholine released.

For guinea-pig trachea there is functional evidence for α_2-adrenoceptor-mediated prejunctional inhibition of cholinergic transmission (Table 7). In view of the sparse adrenergic innervation of airway smooth muscle, it has been suggested that physiological activation of β-adrenoceptors inhibiting acetylcholine release through circulating adrenaline is an additional cause for bronchiolar relaxation to postjunctional adrenergic effects on the smooth muscle, particularly in asthma where cholinergic tone is increased (Danser et al. 1987; Ito 1988; Rhoden et al. 1988). Direct evidence for this view remains to be provided.

With one exception (Danser et al. 1987), indirectly acting amines did not produce adrenoceptor-mediated prejunctional effects but sympathetic nerve stimulation has generally been effective to mimick prejunctional actions of exogenous agonists that, for technical reasons, are more easily studied (Tables 7, 8).

3.3 Dopamine Receptors

Compared with the large body of evidence of prejunctional dopamine receptors in the brain (Chesselet 1984; Langer and Lehmann 1988) and on noradrenergic peripheral nerves (see chapter 2.3), the occurrence of dopamine receptors on peripheral cholinergic neurones has been difficult to establish. Earlier efforts in this direction were hampered by the lack of selective dopamine antagonists and by disregard of the potency of dopamine at prejunctional α- and postjunctional β-adrenoceptors (for a critical review, see Willems et al. 1985).

Vagal cardiac neurotransmission in the pithed rat is inhibited by a D_2 receptor but the site involved (prejunctional and/or ganglionic) cannot be specified (Roquebert et al. 1991). In the guinea-pig ileum most investigators failed to detect modulatory dopamine receptors for acetylcholine release (Table 9). Inhibitory effects of dopamine in the guinea-pig gastrointestinal tract reported by some authors turned out to be mediated by α-adrenoceptors (Görich et al. 1982; see also review of earlier work by Kilbinger and Weihrauch 1982). There is the possible exception of the guinea-pig stomach where D_2 receptors mediate inhibition of acetylcholine release (Kusunoki et al. 1985). Although field stimulation-evoked acetylcholine release from Auerbach's plexus was also not found to be affected by dopamine, the release facilitated by a low concentration of oxotremorine appeared to be inhibited by a pimozide-sensitive dopamine receptor (Das et al. 1991). At larger concentrations of oxotremorine (5 and 10 μM) the well-known muscarinic inhibitory action (Kilbinger 1977) prevailed over facilitation (Das et al. 1991).

3.4 5-Hydroxytryptamine Receptors

Although presently the literature on 5-HT receptors modulating noradrenaline release in the periphery is more voluminous (Table 2) than that on acetylcholine release (see below), it was the latter area of autonomic neurotransmission which originally was brought to attention, leading to important work particularly on the intestine (see review by Kosterlitz and Lees 1964). Gaddum and Picarelli (1957) provided indirect evidence in the guinea-pig ileum for a neuronal 5-HT receptor (the "M receptor", apparently blocked by morphine) mediating acetylcholine release that was responsible for part of the contraction elicited by the amine. Ádám-Vizi and Vizi (1978) directly demonstrated tetrodotoxin-sensitive acetylcholine release from Auerbach's plexus of the

Table 8. Modulation of cholinergic neurotransmitter overflow/neurotransmission by β-adrenoceptors

Species	Tissue	Stimulation + parameter tested	Agonist	Effect on ACh release	Antagonist	Subtype	Comment	Reference
Chicken	Heart (perfused)	VNS, 5 Hz, 30 s ^{3}H overflow	Isoprenaline	None	Propranolol		No alteration of ACh overflow by β-receptor agonist or antagonist	Löffelholz et al. 1984
Rat	Trachea	FS, 15 Hz, 20 s ^{3}H overflow	Isoprenaline Fenoterol and other	Inhibition None	Propranolol, CGP20712A	β_1	Inhibition by isoprenaline (but not β_2-agonists) of ACh overflow prevented by propranolol and the β_1 selective antagonist, as well as by epithelium removal or indomethacin: transsynaptic inhibition	Reinheimer et al. 1993
Rat	Atria	K^+ 100 mM, 2 min ^{3}H overflow	NA	Inhibition	Propranolol		Decrease in evoked ACh overflow (see Table 7) not blocked by propranolol: no inhibitory β-AR	Benkirane et al. 1986
Guinea pig	Ileum	FS, 0.3 Hz ACh (B)	Isoprenaline, adrenaline	None Inhibition	Propranolol, phenoxybenzamine		No effect of isoprenaline; that by adrenaline antagonized by phenoxybenzamine but not by propranolol: no modulatory β-receptor	Kosterlitz et al. 1970
Cat	Trachea in vivo	Spontaneous vagal tone in anaesthetized cat	Endogenous NA released by SNS 20 Hz, 5 s Isoprenaline	Inhibition Inhibition	Propranolol	β β	SNS abolished tracheal segment tension but reduced contraction after ACh by only one-half. SNS effect prevented by propranolol	Baker and Don 1987

Dog	Trachea	FS, 20 Hz, 1–30 pulses Contraction e.j.ps.	Procaterol, isoprenaline	Inhibition	ICI118551	β_2	FS evoked contraction and e.j.ps. depressed by procaterol and isoprenaline, all in the presence of bretylium and indomethacin	Ito 1988
Dog	Trachea	FS, 2 or 10 Hz, 5×1 min, ibid, 2 Hz ACh (C) overflow	NA	None	NA release prevented by guanethidine		No modulation of ACh release by exogenous or endogenous NA acting on β-AR	Martin and Collier 1986
	Bronchi		NA	None				
Dog	Bronchi	FS, 2 Hz, 30 s Contraction	Endogenous NA released by tyramine	Inhibition	Propranolol, metoprolol, ICI118551, rauwolscine	β_1	FS induced contraction (but not effect of exogenous ACh) inhibited by NA released by tyramine in the presence of bretylium and indomethacin. Inhibition prevented by β_1-but not β_2- or α_2-AR blockade	Danser et al. 1987
			Xylazine	None				
Man	Bronchi	FS, 4–32 Hz, 15 s Contraction	Isoprenaline, adrenaline, NA	Inhibition	Propranolol, betaxol, ICI118551	β_2	FS evoked contractions more potently inhibited by catecholamines (ISO>A>NA) than ACh evoked responses. Inhibition prevented by β_2- but not β_1-AR blockade	Rhoden et al. 1988
			Tyramine	None				
Man	Bronchi	FS, 20 Hz, 1.5 s Contraction	Procaterol	Inhibition	ICI118551	β_2	FS evoked (atropine-sensitive) contraction inhibited by procaterol which did not affect response to exogenous ACh	Aizawa et al. 1991

For abbreviations and code names, see Table 7.

Table 9. Modulation of cholinergic neurotransmitter overflow/neurotransmission by dopamine receptors

Species	Tissue	Stimulation + parameter tested	Agonist	Effect on ACh Release	Antagonist	Subtype	Comment	Reference
Rat (pithed)	Heart in vivo	VNS, 3–9 Hz, 15 s Heart rate	Apomorphine, bromocriptine, pergolide, quinpirole	Inhibition	(*S*)-Sulpiride, domperidone, yohimbine, prazosin, SCH23390	DA_2	Atenolol pretreatment; VNS-evoked but not ACh-induced bradycardia was inhibited by agonists; this effect was antagonized by sulpiride or domperidon but not by AR blockers: prejunctional and/or ganglionic DA_2 receptors assumed	Roquebert et al. 1991
Guinea pig	Stomach	FS, 10 Hz, 30 s ^3H overflow	DA, LY171555, SKF38393	Inhibition None	Haloperidol, sulpiride, domperidone, yohimbine, propranolol, ketanserin	DA_2	Decrease of ACh overflow by DA unaffected by hexamethonium, mimicked by LY171555, and inhibited by the DA antagonists. No effect of α_2-, β-AR or HT_2 antagonists	Kusunoki et al. 1985
Guinea pig	Ileum	FS, 0.3 Hz, 5 min ACh (B) overflow	DA Adrenaline, NA	None Inhibition			see Table 7	Paton and Vizi 1969
Guinea pig	Ileum	FS, 0.1 Hz Contraction	DA, apomorphine NA, clonidine	Inhibition	*cis*-Flupenthixol, pimozide, domperidone, metoclopramide, sulpiride, tolazoline		Inhibitory action of DA on twitch response not antagonized by flupenthixol, pimozide or domperidone. Profile of metoclopramide,	Görich et al. 1982

							sulpiride and tolazoline pA_2 values against DA, NA and clonidine suggest neuronal inhibitory α-AR stimulated by DA; no evidence for specific DA receptor	
Guinea pig	Ileum	FS, 0.1 Hz Contraction	Quinpirole, 1–100 n*M* 1–100 μ*M*	None Facilitation	Methysergide, ketanserin, and other		Quinpirole concentrations selective for DA receptors ineffective but larger ones caused facilitation of twitch response unaltered by antagonists of 5-HT-, DA-, opioid, $GABA_A$ or muscarinic receptors; no facilitation of response to ACh or carbachol. Prejunctional facilitation of ACh release assumed, mechanism unclear	Guenaneche and Lefebvre 1990
Guinea pig	Ileum	FS, 1 Hz, 15 min + oxotremorine present ACh (**B**) overflow	DA	Inhibition	Pimozide	DA	FS evoked ACh overflow was facilitated by oxotremorine 125 n*M*; DA 2.5–10 n*M* inhibited facilitated ACh overflow, an effect that was antagonized by pimozide 250 nM; no effect of DA on evoked ACh overflow in the absence of oxotremorine	Das et al. 1991

Table 9 (*Contd.*)

Species	Tissue	Stimulation + parameter tested	Agonist	Effect on ACh Release	Antagonist	Subtype	Comment	Reference
Rabbit	Retina	Superfusion with DA agonists ^{3}H (ACh) overflow Adenylyl cyclase activity	DA, apomorphine, fenoldopam, SKF38393, (*R*) and (*S*) Pergolide	Increase of basal overflow	SCH23390, butaclamol, *cis*- or *trans*-flupenthixol, fluphenazine, and other	DA_1	DA agonists cause Ca-dependent ACh release and stimulate adenylyl cyclase at similar order of potency; also correlation with agonist affinities at ^{3}H-SCH23390 binding sites. Orders of potencies and effects of antagonists characteristic for DA_1 receptor	Hensler et al. 1987

For abbreviations and code names, see Table 7.

longitudinal muscle preparation by 5-HT and showed that morphine blocked release at a site distal to the 5-HT receptive site, rather than being a 5-HT antagonist. Similar findings were later reported for guinea-pig small intestine submucous plexus (Yau et al. 1990). Concerning acetylcholine release in the intestine, Fozard (1984) suggested the presence of at least two different 5-HT receptors on cholinergic neurones. This was confirmed by the following observations by Kilbinger and Pfeuffer-Friederich (1985). 5-HT caused a transient (rapidly desensitizing) enhancement of basal, and a non-desensitizing inhibition of field stimulation-evoked, [^{3}H]-acetylcholine release. The amine had a greater potency at the inhibitory than at the excitatory receptor. While ketanserin left both responses unaltered, methiothepin competitively blocked the inhibitory but not the excitatory action, indicating that a 5-HT_1 receptor mediated the inhibition, and a non-5-HT_1, non-5-HT_2 receptor, the excitation. A more precise classification of the inhibitory receptor was possible after development of the selective agonist, 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT). Using this drug, Fozard and Kilbinger (1985) classified the receptor inhibiting stimulation-evoked [^{3}H]-acetylcholine release and the accompanying contraction response of guinea-pig ileum as 5-HT_{1A}.

Recent progress concerns the facilitatory action on acetylcholine release which has to be attributed to two different receptors, 5-HT_3 and 5-HT_4. First, 5-HT_3 receptor involvement was ascertained by Fox and Morton (1990) who showed that ondansetron (GR38032F), a highly selective antagonist (Butler et al. 1988), greatly attenuated the facilitatory response of 10 μM 5-HT on basal [^{3}H]-acetylcholine overflow. Ondansetron yielded pA_2 values of 7.62 and 7.64 against 5-HT (micromolar concentrations) and 2-methyl-5-HT, a selective 5-HT_3 receptor agonist, respectively, which agreed with those obtained with the same agonists for guinea-pig ileum longitudinal muscle contraction responses (Butler et al. 1988). However, Craig and Clarke (1990) had noted that the ability of submicromolar concentrations of 5-HT to enhance the twitch response to submaximal electrical stimulation could not be blocked by ondansetron but by a large concentration of tropisetron at a pA_2 value of 6.5, which distinguished the receptor from the 5-HT_3 site for which tropisetron has a pA_2 value of 8.0. Craig and Clarke (1990) suggested the presence of a different receptor in the ileum, 5-HT_4, and showed the selective agonist potency of 5-methoxytryptamine at this site. Subsequently, Kilbinger and Wolf (1992), determining [^{3}H]-acetylcholine release from guinea-pig myenteric plexus, found that the effect of 5-methoxytryptamine was antagonized by tropisetron at a pA_2 value

of 6.6, which is characteristic for the 5-HT_4 receptor. On the other hand, tropisetron antagonized the low-affinity (micromolar and above) component of the concentration-response curve of 5-HT at a pA_2 value of 8.0, in line with a 5-HT_3 receptor-mediated effect. Both 5-HT and 5-methoxytryptamine, after eliciting the transient elevation of basal [^{3}H]-acetylcholine overflow, caused a concentration-dependent inhibition of field stimulation-evoked [^{3}H]-acetylcholine release. Tropisetron reinforced the inhibitory effect of 5-methoxytryptamine, whereas methiothepin reversed the inhibition to a facilitation of evoked [^{3}H]-acetylcholine release, suggesting inhibition via 5-HT_1 and facilitation via 5-HT_4 receptors, respectively. Thus, the facilitatory action of 5-HT_4 receptor activation was revealed only when the predominant inhibitory action was blocked (Kilbinger and Wolf 1992). Unlike 5-methoxytryptamine, the novel 5-HT_4 receptor agonist BIMU 8 (see Tonini et al. 1991), which lacks 5-HT_1 receptor activity, caused enhancement of field stimulation-evoked [^{3}H]-acetylcholine release in the absence of methiothepin, and its effect on [^{3}H]-acetylcholine release was antagonized by the selective 5-HT_4 blocker, DAU 6285 (Kilbinger et al. 1992b).

When functional, biochemical and much electrophysiological evidence is incorporated into a "wiring" diagram of myenteric neurones, the facilitatory 5-HT_3 receptor is probably located on the somadendritic portions of cholinergic motor neurones and excitatory cholinergic interneurones, as well as on the somata of inhibitory non-cholinergic interneurones (Tonini et al. 1991). According to this scheme, the terminals of the cholinergic neurones bear both excitatory 5-HT_4 receptors, mediating the increase in acetylcholine release and prokinetic action elicited by a number of drugs, and the inhibitory 5-HT_{1A} receptors whose physiological function still needs to be defined.

There are no reports of measurements of acetylcholine release in the airways modulated through 5-HT receptors. Functional studies have shown that contractions of rat bronchi in vitro (Szarek et al. 1993) and guinea-pig bronchi in vivo (Macquin-Mavier et al. 1991) that are elicited by electrical stimulation of cholinergic nerves are facilitated by 5-HT acting on a 5-HT_2 receptor. These effects could be differentiated from a purely postjunctional combined action of acetylcholine and 5-HT. It might also be of interest that in a study on electrically stimulated human urinary bladder strips, 5-HT exerted neuronal inhibitory actions via a 5-HT_1 receptor, and facilitatory actions not to be explained by 5-HT_{1-3} subtypes but what now appears to be characteristic for the 5-HT_4 receptor (Corsi et al. 1991).

3.5 Histamine Receptors

In guinea-pig ileum segments exposed to pyrilamine (to block H_1 receptor-mediated contractions), histamine or N-methylhistamine inhibited electrically evoked twitch responses, an action competitively blocked by the H_3 antagonist impromidine but not by a variety of other receptor blocking agents including cimetidine (Trzeciakowski 1987). The inhibitory H_3 receptor resembled that previously characterized for histamine autoreceptors in brain (Schwartz et al. 1986) by a similar pA_2 value of impromidine and the relative potencies of the two agonists. A prejunctional effect was assumed, as N^{α}-methylhistamine did not alter the ileal response to exogenous acetylcholine, and tetrodoxin blocked the relaxation. The inhibitory H_3 receptor was confirmed by Poli et al. (1991) who found that both histamine and (*R*)-α-methylhistamine concentration dependently decreased [^{3}H]-acetylcholine release from guinea-pig ileum myenteric plexus stimulated at 1 Hz for 3 min, while H_1 and H_2 receptor blockers were present. Impromidine and thioperamide antagonized the inhibition and facilitated evoked acetylcholine release in the absence of exogenous histamine, supposedly by antagonizing endogenous histamine released from mast cells by electrical stimulation. A prejunctional H_3 receptor suppressing nicotinic transmission in the submucous ganglia of guinea-pig colon and which may be activated by amine release from allergen-sensitized mast cells was suggested from electrophysiological evidence by Frieling et al. (1993).

H_3 receptor-mediated inhibition of cholinergic neurotransmission causing contraction of the guinea-pig trachea in vitro was reported by Ichinose et al. (1989). However, cholinergic bronchoconstriction caused by electrical stimulation of the dorsal medulla in anaesthetized guinea pigs was unaffected by H_3 receptor activation, whereas histamine or (*R*)-α-methylhistamine potentiated bronchoconstriction through a mechanism involving H_1 receptors (Hey et al. 1992b). In the airways, the pathophysiological significance for histaminergic modulation of acetylcholine release is obvious, and the effects of endogenously released histamine should further be studied. Histamine and 5-HT release from mast cells after infusion of compound 48/80 occurs in the rat perfused heart and is associated with a 100% facilitation of the [^{14}C]-acetylcholine release evoked by 10 Hz vagus nerve stimulation 4 min after infusion of 48/80 (Fuder et al. 1994). It was suggested that the facilitation, which only partly appeared to be due to histamine release, was mediated by an H_3 receptor because it was reduced to about half by thioperamide, but not by mepyramine or cimetidine.

3.6 Adenosine Receptors/Purinoceptors

The inhibitory effect on acetylcholine release and cholinergic neurotransmission by adenosine and related nucleotides as an event mediated by prejunctional receptors was first demonstrated by Vizi and Knoll (1976) on the guinea-pig ileum. These observations were confirmed and extended under a variety of experimental conditions (Table 10) showing that both exogenous and endogenously formed adenosine acting on an A_1 receptor decreases the acetylcholine release evoked by a low stimulation frequency, but hardly that evoked by a higher one. ATP and other nucleotides act after hydrolysis to adenosine. The prejunctional effects are enhanced by adenosine uptake or deaminase inhibitors, and are unrelated to agonist actions on cAMP-phosphodiesterase or adenylyl cyclase (for review of earlier papers, see Vizi 1979, Fredholm and Hedqvist 1980, Gustafsson 1980). In some organs (rat bronchi, rabbit bronchi and atria) facilitation of acetylcholine release has been reported, possibly mediated by an A_2 receptor (Table 10).

Up to about 1990, ATP was not thought to have prejunctional modulatory effects on its own (Olsson and Pearson 1990), although there was a suggestion of a nucleotide action per se on a purinoceptor that cannot distinguish between nucleosides and nucleotides (Wiklund et al. 1985). However, facilitation of acetylcholine release in the guinea-pig ileum by the stable analogue, α,β-methylene-ATP, has been reported to be mediated via a P_{2X} receptor (Sperlagh and Vizi 1991). Lack of a facilitatory action of α,β-methylene-ATP in experiments by Katsuragi et al. (1993) may have been due to the presence of eserine and the fact that the stimulation conditions were more vigorous than those of Sperlagh and Vizi (1991). According to the latter authors, ATP has opposing effects: facilitatory ones via P_{2X} receptors and inhibitory ones after hydrolysis to adenosine mediated by A_1 receptors. The net effect will, therefore, depend on the activity of ectonucleotidases in the vicinity of receptor and release sites. It remains to be seen whether the facilitatory ATP effects on acetylcholine release correspond to the excitatory effects of ATP on sympathetic, parasympathetic and enteric neurones observed in electrophysiological experiments (see Illes and Nörenberg 1993); one complicating fact for equating these actions is the apparent mediation by P_{2Y} receptors of the electrophysiological effects of ATP.

The prejunctional adenosine effects are not due to interaction with adrenergic prejunctional mechanisms (Gustafsson et al. 1987). Particularly, the facilitatory effect on acetylcholine release of P_{2X} receptor activation by α,β-methylene-ATP cannot be explained by

interruption of a noradrenergic inhibitory tone, as noradrenaline release itself is enhanced rather than decreased by the drug (Sperlagh and Vizi 1991). Acetylcholine release from synaptosomes evoked by field stimulation (Reese and Cooper 1982; Shinozuka et al. 1985) or by nicotinic drugs (Reese and Cooper 1982) is more susceptible to inhibition by adenosine than that evoked by high potassium. This and other evidence (see Table 10) suggests that adenosine inhibits calcium influx into the neurone. For example, the inhibitory adenosine effect is antagonized by external calcium ion elevation or the calcium channel opener, BAY k 8644 (Katsuragi et al. 1990). However, calcium channel modulators cause effects on neurosecretion also in the absence of adenosine receptor agonists and additive actions or a functional antagonism cannot be excluded.

3.7 Prostanoid and Leukotriene Receptors

In view of earlier conflicting observations regarding the presence or absence of modulatory effects of PGs on cholinergic transmission in the guinea-pig ileum, Gustafsson et al. (1980) re-investigated the question and determined acetylcholine overflow by a chemical method. On the guinea-pig ileum and the bovine iris, PGE_2 enhanced the contractions evoked by field stimulation and exogenous acetylcholine in a similar manner. Furthermore, basal or stimulated acetylcholine release from the ileum (0.5 or 3 Hz) and the iris (3 Hz) was unaffected by PGE_2. Indomethacin rapidly and nearly completely inhibited evoked contractions, but acetylcholine overflow was decreased very slowly and after 60 min by only 40%. Subsequent PGE_2 administration caused a slight increase of evoked acetylcholine release, but not a reversal to control values. Gustafsson et al. (1980) concluded that a PGE_2-mediated enhancement of acetylcholine release, if at all present, is considerably less important than a postjunctional prostanoid effect. It was, however, recognized that the interpretation of observations on acetylcholine overflow is complicated by the presence of eserine in the incubation media.

The following observations reveal effects of endogenous prostanoids on the excitability of mesenteric ganglia stimulated by exogenous agonists, rather than modulation of acetylcholine release caused by direct excitation of terminal nerve fibres. According to Yagasaki et al. (1984) potassium-induced acetylcholine release was unaffected by indomethacin, but that evoked by nicotinic drugs or by substance P was found to be decreased; PGE_2 partly reversed the inhibition but had no

Table 10. Modulation of cholinergic neurotransmitter overflow/neurotransmission by adenosine receptors/purinoceptors

Species	Tissue	Stimulation + parameter tested	Agonist	Effect on ACh release	Antagonist	Subtype	Comment	Reference
Chicken	Oesophagus	Drug addition Mechanical response	ATP	Stimulation	Theophylline, atropine	A	TTX and atropine sensitive contraction after ATP antagonized by theophylline: Excitatory action of ATP breakdown product on cholinergic neurone	Mishra and Raviprakash 1988
Rat	Bronchi	K^+ 51 m*M*, 5 min ^3H (ACh) overflow	Adenosine	Inhibition	8-PT	A	Experiments carried out at 25°C. Decrease in ACh overflow blocked by 8-PT	Aas and Fonnum 1986
Rat	Bronchi	K^+ 51 m*M*, 5 min ^3H overflow at 25°C	NECA CGS21680 *R*(−)-PIA	Facilitation None None	8-PT	A_2-like	NECA but not the A_2 selective agonist, CGS21680, or *R*(−)-PIA increased K^+ evoked ^3H overflow (prolonged duration of facilitation). NECA antagonized by 8-PT	Waldey and Aas 1991
Rat	Heart (in vivo)	VNS, 1.6–12.8 Hz, 15 s Heart rate	Adenosine	Inhibition	Theophylline	A	Bradycardic effect of VNS inhibited by adenosine infused into right atrium. Theophylline antagonized inhibitory effect. Bradycardia caused by exogenous ACh was enhanced by adenosine	Monteiro and Ribeiro 1991
Guinea pig	Trachea	FS, 20 Hz, 1 s	Adenosine	None			No inhibitory effect of adenosine on FS evoked (and atropine-sensitive)	Grundström et al. 1981a

		Contraction					contractions under conditions where NA decreased them by α_2-AR stimulation	
Guinea pig	Ileum	FS, 0.2 or 10 Hz, cholecystokinin 0.1 μM ACh (B) overflow	Adenosine, ATP	Inhibition	Theophylline	P_1	FS (0.2 Hz) evoked overflow was decreased by adenosine or ATP, an effect blocked by theophylline. On its own, theophylline enhanced evoked overflow. At 10 Hz, ATP was ineffective. Cholecystokinin evoked overflow also inhibited by adenosine. Twitch responses to FS 0.1 Hz decreased by adenosine, ATP, ADP and AMP. Sensitivity of muscle to exogenous ACh unaltered. Competitive blockade by theophylline of adenosine effect	Vizi and Knoll 1976
		Contraction	Adenosine, ATP, ADP, AMP	Inhibition	Theophylline	P_1		
Guinea pig	Ileum	FS, 0.2 Hz Contraction	Adenosine, ATP β,γ-meATP	Inhibition	Theophylline	P_1	ATP or β,γ-meATP decreased twitch response to FS, as did adenosine (EC_{50}, 0.43 μM). Adenosine effect blocked by theophylline and enhanced by dipyridamole. All agonist effects decreased by adenosine deaminase: mediation by adenosine as ATP breakdown product	Moody and Burnstock 1982
Guinea pig	Ileum	FS, 3 Hz, 2.7 min	R(−)-PIA, NECA, S(+)-PIA	Inhibition		A_1 A_2?	Decrease of ACh overflow similar after NECA or $R(-)$- and $S(+)$-PIA.	Gustafsson et al. 1985

Table 10 (*Contd.*)

Species	Tissue	Stimulation + parameter tested	Agonist	Effect on ACh release	Antagonist	Subtype	Comment	Reference
		ACh (C) overflow Contraction	Adenosine, CADO, R(−)-PIA, NECA	Inhibition			Potency ratio for inhibition of contraction: CADO = NECA= R(−)-PIA > adenosine. Conclusion: Adenosine inhibits transmission mainly by prejunctional A_1 receptor; supplementary activation of postjunctional A_2 and possibly prejunctional A_2 receptor	
Guinea pig	Ileum	FS, 2 Hz, 15 min ACh (C) overflow	Adenosine	Inhibition	Theophylline	P_1	Decrease of ACh overflow by adenosine antagonized by theophylline or Bay k 8644, and enhanced by halving external Ca^{2+}. Conclusion: Adenosine acts via neuronal Ca^{2+} channels	Katsuragi et al. 1990
Guinea pig	Ileum	FS, 1 Hz, 3 min ^3H overflow	Adenosine	Inhibition	Theophylline	P_1	Decrease of overflow after adenosine or by hypoxia enhanced by dipyridamole and antagonized by theophylline. Conclusion: Effect of hypoxia partly due to enhanced endogenous adenosine formation	Milusheva et al. 1990

Guinea pig	Ileum	FS, 1 Hz 3 min ^{3}H overflow	Adenosine, ATP, α,β-meATP 2-Methyl-2-thio-ATP	Inhibition Inhibition Facilitation None	8-PT	P_1 P_{2X}	Decrease by P_1 receptor stimulation, and increase by P_{2X} receptor stimulation, of evoked ACh overflow. P_{2Y} agonist inactive. 8-PT increased overflow suggesting inhibitory tone of endogenous adenosine	Sperlagh and Vizi 1991
Guinea pig	Ileum	FS, 3 Hz, 15 min ACh (C) overflow ATP overflow	α,β-meATP β,γ-meATP	Inhibition	Theophylline Suramin for ATP release	P_1 P_{2X}	Decrease of FS evoked overflow by α,β-meATP blocked by theophylline or suramin; α,β-meATP causes suramin sensitive release of ATP. Conclusion: Transsynaptic inhibition by adenosine formed from ATP	Katsuragi et al. 1993
Guinea pig	Ileum submucosal neurones	Focal electrical stimulation Cell membrane potential, nicotinic e.p.s.p.	Adenosine, CADO, NECA, R(−)-PIA, CGS21680 and other	Depolarization (postjunctional) Inhibition of e.p.s.p. (prejunctional)	CPT	A_2 A_1	Rank order of potency of adenosine and congeners indicates depolarization of submucosal neurones via A_2 receptor, and inhibition of ACh release from intramural nerves by A_1 receptor activation; both competitively antagonized by CPT. Responses to applied ACh unaltered by CADO	Barajas-Lopez et al. 1991
Guinea pig	Ileum myenteric	K^+ 50 m*M*, 2 min,	Adenosine, ATP	Inhibition	Theophylline	P_1	Decrease of ACh overflow by adenosine larger when	Reese and Cooper 1982

Table 10 (*Contd.*)

Species	Tissue	Stimulation + parameter tested	Agonist	Effect on ACh release	Antagonist	Subtype	Comment	Reference
	plexus synaptosomes	DMPP, 2 min ^{3}H ACh overflow					evoked by DMPP than by K^+. Inhibition by both agonists of nicotinic release reversed by theophylline	
Guinea pig	Ileum myenteric plexus synaptosomes	K^+ 40 m*M*, 10 min, FS, 10 Hz, 5 min ^{3}H -ACh overflow ^{45}Ca uptake	Adenosine	Inhibition			Ca-dependent ACh overflow evoked by K^+ partially, and FS evoked release completely, inhibited by adenosine. Depolarization evoked ^{45}Ca uptake by synaptosomes also inhibited by adenosine	Shinozuka et al. 1985
Rabbit	Iris sphincter	FS, 3–10 Hz, 10–30 s Contraction	Adenosine, *R*(−)-PIA, CADO, NECA	Inhibition	8-pST	A_1	Adenosine and other agonists inhibited phase I contraction (cholinergic) at potency ratios suggesting prejunctional A_1 receptor. Endogenous inhibitory A_1 tone indicated by increase of phase I contraction after 8-pST	Gustafsson and Wiklund 1986
Rabbit	Bronchi	FS, 5–10 Hz, 10 s Contraction	Adenosine, R(−)-PIA, NECA	Facilitation	8-pST	A_2	Potency order of adenosine derivatives to enhance FS-evoked contraction indicative of prejunctional excitatory A_2 receptor. No facilitation of responses to exogenous ACh	Gustafsson et al. 1987

Rabbit	Atria (perfused)	VNS, 2 Hz, 3 min ^{14}C-ACh overflow NA (C) overflow	SNS (2 Hz, 3 min) evoked cotransmitter release	Facilitation	CP66713	A_2?	When SNS pulses were applied 19 ms before VNS pulses, ACh overflow was increased (reserpine-resistant effect); partial inhibition by selective A_2 antagonist, CP66713	Muscholl and Habermeier-Muth 1992
Dog	Heart in vivo	VNS, 20 Hz, ERP	Adenosine	Inhibition			Adenosine decreased ERP response to VNS, as did hypoxia, but not ERP response to methacholine	Miyazaki and Zipes 1990

α,β-meATP, β,γ -meATP, α,β- or β,γ-methylene-ATP; CADO, 2-chloroadenosine; *R*(−)-PIA, *R*(−)-N^6-(2-phenylisopropyl)-adenosine; S(+)-PIA, S(+)-N^6-(2-phenylisopropyl)-adenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; 8-PT, 8-phenyltheophylline; 8-pST, 8-p-sulphophenyltheophylline; ERP, effective refractory period; CPT, 8-cyclopentyltheophylline.

For other abbreviations, see Table 7.

effect on its own (Yagasaki et al. 1984; Takeuchi et al. 1991). A complete reversal by a stable prostacyclin analogue under identical experimental conditions was reported by Fukunaga et al. (1993); however, PGD_2, PGF_2 and a thromboxane A_2 analogue did not reverse the indomethacin effect. Moreover, the spontaneous release from the ileum of 6-keto-PGF_2, a stable prostacyclin metabolite, exceeded that of PGE_2 by a factor of 3–5; the release of neither prostanoid was changed by nicotine. Therefore, Fukunaga et al. (1993) concluded that prostacyclin rather than another prostanoid is the endogenous modulator of nicotinic or substance P-induced acetylcholine release in the guinea-pig ileum. However, the acetylcholine release evoked by electrical field stimulation was sensitive to inhibitors of 5-lipoxygenase, and metabolites of arachidonic acid produced by the 5-lipoxygenase pathway (5-hydroxyeicosatretraenoic acid, leukotrienes C_4, D_4, E_4) reversed the inhibition (Yoshikawa et al. 1993). All these experiments were done in the presence of eserine.

Airways have been considered to be another organ system in which prostanoids are likely candidates for a neuromodulatory role in cholinergic pathways, as their rate of release is remarkably high (Yamaguchi et al. 1976; Walters et al. 1984) compared with the concentrations of exogenous PGE_2 required for mechanical effects (see also review by Barnes 1992). Several authors have shown an inhibitory effect of endogenous prostanoids or exogenous PGE_2 on acetylcholine overflow from rat (Reinheimer et al. 1993), guinea-pig (Wessler et al. 1990) and dog trachea (Shore et al. 1987) or bronchi (Deckers et al. 1989); dog tissue experiments were done in the presence of cholinesterase inhibitors. These observations are in line with functional studies reporting a greater inhibitory effect of exogenous PGE_2 on field stimulation-evoked than on acetylcholine-evoked smooth muscular contraction of guinea-pig (DeLisle et al. 1992), canine (Walters et al. 1984; Ito 1991) and equine trachea (Tessier et al. 1991). Prostacyclin was found to be far less effective than PGE_2 (Shore et al. 1987). It has been suggested that the increase in bronchial tone observed after administration of non-steroidal anti-inflammatory drugs may at least partly be due to an increase in cholinergic tone as a result of impaired inhibition of local prostaglandin formation (Deckers et al. 1989).

On the other hand, dissenting views do exist concerning a predominant postjunctional role of prostaglandins. As was observed by Gustafsson et al. (1980) for the facilitatory prostaglandin effect in the ileum, there is a temporal dissociation between the immediate inhibitory PGE_2 effect on field stimulation-evoked contractions and the

delayed one on acetylcholine overflow also in the dog trachea (Shore et al. 1987). Furthermore, noting a subsensitivity of smooth muscle to exogenous acetylcholine produced by PGE_2, the latter authors pointed out that despite a proven prejunctional effect of prostanoids in the dog airways, the postjunctional action is the functionally more important one.

In contrast to the ileum where a thromboxane A_2 analogue does not appear to affect cholinergic transmission (see above), there is positive evidence of a prejunctional facilitatory action of another one (U46619) in canine bronchial or tracheal muscle (Chung et al. 1985; Serio and Daniel 1988; Janssen and Daniel 1991); this evidence is derived from the fact that there is enhancement of field stimulation-evoked contraction but an unaltered response to exogenous methacholine, acetylcholine or carbachol, respectively. In addition, contractions induced by efferent vagus nerve stimulation were augmented by U46619 (Munoz et al. 1986). Similarly, PGD_2, which is released by human lung mast cells after antigen challenge, has been reported to enhance contraction of dog bronchi to electrical field stimulation but not to added acetylcholine (Tamaoki et al. 1987). However, PGD_2 did not alter basal acetylcholine release or that evoked by 5 Hz stimulation from canine bronchi (Deckers et al. 1989); and Serio and Daniel (1988) failed to observe any effect of PGD_2 on field stimulation-evoked contractions. Leukotrienes C_4 and D_4 had no effect on cholinergic neurotransmission in canine trachea (Inoue and Ito 1985; Serio and Daniel 1988).

We found no recent reports dealing with prostanoid effects on cholinergic transmission in the heart. Previously Wennmalm and Hedqvist (1971) had shown that PGE_1 decreased the bradycardic effect of vagus nerve stimulation in the perfused rabbit heart without affecting the response to exogenous acetylcholine. Similar observations were made by Feniuk and Large (1975) with PGE_1 and PGE_2 in the anaesthetized mouse.

Summing up, most evidence indicates that PGE_2 has a facilitatory action on acetylcholine release in ileum and induces inhibition in airways and heart. Thromboxane A_2 receptor activation causes facilitation of release in dog airways but fails to affect release in the ileum. However, in the majority of experiments with acetylcholine determinations, a cholinesterase inhibitor had been added to the incubation media. Furthermore, one complication of nerve or field stimulation in evaluating prejunctional mechanisms of prostanoids is the continuing or even increasing release of the compounds due to mechanical activity during experiments in ileum (Botting and Salzmann 1974), heart (Junstad and

Wennmalm 1974) and airways (Yamaguchi et al. 1976; Walters et al. 1984; Ito 1991). Therefore, future studies with more refined techniques are needed to clarify the role of prejunctional prostanoid receptors for cholinergic transmission.

3.8 Amino Acid Receptors

In the mammalian brain, the inhibitory GABA receptor which is coupled to chloride conductance control can be activated selectively by muscimol or blocked by bicuculline. In an attempt to antagonize GABA-mediated inhibitory effects on peripheral autonomic nerves, Bowery et al. (1981) noticed the failure of bicuculline to antagonize the response to GABA and proposed a different receptor, later to be named $GABA_B$, as responsible for prejunctional control of noradrenaline release in rat atrium (see Sect. 2.10) and cholinergic transmission in guinea-pig ileum, as opposed to the original ($GABA_A$) receptor found in the brain. With refinement of receptor classification, the occurrence of $GABA_B$ sites was also established for the central nervous system, where considerable regional variations of both receptor densities exist (Bowery 1993).

GABAergic effects have been observed in various peripheral organs (Erdö and Bowery 1986). Both inhibitory actions (as expected from work on brain) and excitatory ones are known. In particular, acetylcholine–GABA interactions are likely to occur in those organs where both autacoids serve neurotransmitter functions such as retina (Hutchins 1987), gastrointestinal tract (Jessen et al. 1987) or urinary bladder (Santicioli et al. 1986).

In the rabbit retina in vivo (eye-cup preparation), the release of [^{3}H]-acetylcholine evoked by light flashes is inhibited via a $GABA_A$ receptor mechanism. This has been observed after local application of GABA or muscimol (Massey and Neal 1979; Cunningham and Neal 1983), or by elevating the endogenous GABA concentration with inhibitors of its uptake (Neal et al. 1992); these GABAergic effects were antagonized by bicuculline or picrotoxin, a non-competitive $GABA_A$ receptor blocker. Similarly, on isolated rabbit retina, dopamine-induced [^{3}H]-acetylcholine release was decreased by GABA or muscimol, an effect also antagonized by bicuculline or picrotoxin (Hensler and Dubocovich 1988). The presence of an endogenous GABAergic tone in the retina under basal conditions is indicated by the effectiveness of $GABA_A$ receptor antagonists to enhance spontaneous [^{3}H]-acetylcholine overflow in the rabbit

(Massey and Neal 1979; Cunningham and Neal 1983; Hensler and Dubocovich 1988) or the chick retina (Agardh 1986). Potassium-evoked [^{3}H]-acetylcholine release has not been found to be modulated by exogenous GABA (Agardh 1986). This might be due to the pronounced increase of endogenous GABA released under this condition (Neal et al. 1992), which possibly leads to maximal occupation of GABA receptors. Light-evoked acetylcholine release was facilitated by (-)-baclofen, a selective $GABA_B$ receptor agonist, but not by the inactive (+)-isomer (Cunningham and Neal 1983). This raises the possibility that exogenously applied GABA stimulates both $GABA_A$ and $GABA_B$ receptors in the retina with opposing actions on acetylcholine release, explaining the very low potency of GABA compared with that of muscimol observed by Cunningham and Neal (1983).

In the guinea-pig ileum, GABA inhibits the twitch response via a $GABA_B$ receptor (see above; Bowery et al. 1981). On the field-stimulated myenteric plexus-longitudinal muscle preparation, $GABA_A$ receptor antagonists did not prevent the relaxant effect of GABA and muscimol did not mimic it (Kaplita et al. 1982). This was confirmed by Kleinrok and Kilbinger (1983) who also found that GABA produced a concentration-dependent inhibition of [^{3}H]-acetylcholine release evoked by 1 Hz field stimulation, which corresponded to the mechanical effects, and that the action of GABA on [^{3}H]-acetylcholine release was not antagonized by bicuculline or picrotoxin. Furthermore, the contraction of guinea-pig ileum in response to GABA administration, which is tetrodotoxin-sensitive and is antagonized by bicuculline or picrotoxin (Krantis and Kerr 1981), was clearly related to an increase in basal release of [^{3}H]-acetylcholine; the latter was inhibited by the antagonists in the same fashion to the mechanical responses observed (Kleinrok and Kilbinger 1983). $GABA_A$ receptor-mediated and tetrodotoxin-sensitive or Ca^{2+}-dependent enhancement of [^{3}H]-acetylcholine overflow produced by exogenous GABA or muscimol was additionally reported for guinea-pig ileum (Taniyama et al. 1988) or gall bladder (Saito et al. 1984) and rat antral mucosa/submucosa strips (Harty et al. 1991). Similarly, endogenous GABA released by neuropeptides has been found to be involved in causing [^{3}H]-acetylcholine release on guinea-pig ileum, e.g. after substance P (Tanaka and Taniyama 1985), somatostatin (Takeda et al. 1989) and neurotensin (Nakamoto et al. 1987). This release is mediated via $GABA_A$ receptor stimulation, as indicated by the antagonistic effect of bicuculline. In the case of somatostatin, a facilitatory action of phaclofen, a selective $GABA_B$ receptor antagonist, on evoked acetylcholine release has been reported, which suggests a minor $GABA_B$-

mediated inhibition opposing the major $GABA_A$-mediated stimulatory response (Takeda et al. 1989).

Interesting observations on the superfused guinea-pig inferior mesenteric ganglion have been reported by Parkman et al. (1993). Stimulation at 2 Hz of afferent lumbar colonic nerves caused [^{3}H]-acetylcholine release which was facilitated both by exogenous GABA or muscimol and by diazepam, which has no intrinsic activity by its own but allosterically increases the $GABA_A$-mediated response when the $GABA_A$ receptor is activated. The facilitation of [^{3}H]-acetylcholine release was inhibited by bicuculline or picrotoxin but not by phaclofen. Contrary to $GABA_A$ modulated release of acetylcholine from colonic nerves that evoked by stimulation of central splanchic nerves was inhibited by $GABA_B$ receptor agonists and remained unaffected by muscimol or diazepam. Since release of [^{3}H]-GABA by electrical colonic nerve stimulation or colonic distension was also observed under the experimental conditions, it has been suggested that endogenous GABA liberated from mechanosensory nerve fibres facilitates cholinergic mechanosensory transmission in the ganglion via $GABA_A$ sites, and inhibits central cholinergic transmission via $GABA_B$ sites.

Transmural stimulation of guinea-pig urinary bladder strips causes a cholinergically mediated contraction which is inhibited by GABA, in contrast to a non-cholinergic component of response. The inhibitory action was antagonized by bicuculline or furosemide, a chloride channel blocker, suggesting the presence of $GABA_A$ receptors inhibiting evoked acetylcholine release (Taniyama et al. 1983). Field stimulation of guinea-pig urinary bladder strips has been shown to elicit a Ca^{2+}-dependent and tetrodotoxin-sensitive [^{3}H]-GABA release, and the electrically evoked [^{3}H]-acetylcholine release to be enhanced by bicuculline and furosemide, suggesting inhibitory $GABA_A$ receptors being activated by endogenous GABA (Kusunoki et al. 1984). Similarly, GABA and muscimol but not baclofen reduced both [^{3}H]-acetylcholine release and contractions evoked by nicotine, and these effects were antagonized by bicuculline and furosemide (Kusunoki et al. 1984). The inhibitory effect of GABA released by substance P on [^{3}H]-acetylcholine overflow and the antagonism by bicuculline (Shirakawa et al. 1989) has been mentioned in Sect. 3.11. Thus, in guinea-pig urinary bladder the available evidence points to a $GABA_A$ receptor activated by the endogenous amino acid and inhibiting evoked acetylcholine release. On the other hand, indirect evidence suggests an inhibitory $GABA_B$ receptor in mouse, newborn rat and rabbit urinary bladder (Santicioli et al. 1986).

On guinea-pig airways an inhibitory $GABA_B$ receptor has been classified, by means of various selective agonists and antagonists, which regulates neuronally evoked cholinergic and tachykinin-mediated bronchiolar contraction (Chapman et al. 1993a,b). This is true both for in vivo and in vitro functional responses. Although an increase of basal [^{3}H]-acetylcholine overflow from guinea-pig lung strips after $GABA_A$ receptor agonists has been observed which was Ca^{2+} dependent and tetrodotoxin-sensitive, and although potassium-evoked [^{3}H]-acetylcholine release was inhibited by GABA or baclofen (Shirakawa et al. 1987), endogenous GABA does not appear to mediate contraction of airway smooth muscle, as $GABA_A$ agonists or $GABA_B$ antagonists fail to alter bronchiolar tone (Chapman et al. 1993a,b).

The inhibitory actions of GABA on neurotransmitter release mediated by $GABA_A$ sites are usually explained by the increase in chloride conductance which leads to hyperpolarization and reduced excitability of neurones. $GABA_B$ receptor-mediated inhibition of acetylcholine release in the myenteric plexus is supposed to be due to a block of calcium inward current at the transmitter release site (Cherubini and North 1984), in agreement with the observation of the calcium dependence of baclofen-modulated evoked ileal contractions (Ong et al. 1987). On first sight it might be surprising that basal and evoked acetylcholine release is *facilitated* by the $GABA_A$ receptor, which is linked to a chloride channel (see the antagonistic effects of furosemide shown by Saito et al. 1984; Shirakawa et al. 1987; Taniyama et al. 1988). The reason that depolarization rather than hyperpolarization occurs is that under resting conditions the chloride equilibrium potential E_{Cl} in ganglion cells is lower than the actual membrane potential E_M, and that on increase in chloride conductance E_M approaches E_{Cl} (Bowery and Hill 1986).

Summing up, inhibition of evoked acetylcholine release is mediated by a $GABA_A$ receptor in rabbit retina and guinea-pig urinary bladder. By contrast, in other organs such as guinea-pig airways, intestine and mesenteric ganglion, a $GABA_B$ receptor mediates inhibition, whereas facilitation of basal and evoked acetylcholine overflow is mediated via a $GABA_A$ receptor.

The glutamate receptors of the retina modulating acetylcholine release from the amacrine cells have been the aim of several studies. Exposure of the rabbit retina in vivo (eye-cup method) to glutamate, kainate, quisqualate or NMDA (in the absence of Mg^{2+}) increased basal [^{3}H]-acetylcholine overflow several fold and inhibited the release evoked by light flashes (Cunningham and Neal 1985). Except for kainate, the inhibitory actions were seen only when basal overflow was elevated. Linn

et al. (1991) also observed large increases of [^{3}H]-acetylcholine basal overflow after administration of several glutamate analogues. Low concentrations of kynurenic acid and 6,7-dinitroquinoxaline-2,3-dione (DNQX) which selectively blocked kainate receptor responses abolished the light-evoked release of [^{3}H]-acetylcholine . These observations and additional ones excluding NMDA receptors (Linn and Massey 1991) suggest that the physiological input to the cholinergic amacrine cells liberating acetylcholine is mediated by the kainate receptor (Linn et al. 1991).

3.9 Nitric Oxide and Vasoactive Intestinal Peptide Receptors

Shortly after the identification of NO as a mediator of endothelium-dependent relaxation of vascular smooth muscle, further evidence accumulated that NO might be involved in various physiological functions, including in neurotransmitter pathways or even as a neurotransmitter proper in the central as well as the autonomic nervous systems (see reviews by Moncada et al. 1991; Sanders and Ward 1992; Sneddon and Graham 1992). Notably, the Ca^{2+}-calmodulin-dependent isoform I of the NO synthase is present in the brain and various peripheral nerves (see Förstermann et al. 1993). A number of biochemical tools are available to evaluate the functional role of endogenously formed NO by the L-arginine–NO pathway, including:

- Competitive inhibitors (e.g. N^G-monomethyl- or N^G-nitro-L-arginine and N^G-nitro-L-arginine methyl ester) of NO synthases
- Reversal of NO synthase inhibition by L- but not by D-arginine
- Imitation by exogenous nitrodilators or NO itself of the expected response to endogenous NO that should not be affected by synthase inhibitors
- Inactivation by oxyhemoglobin or prolongation of the half-life of NO by superoxide dismutase

In the following studies, the possible involvement of endogenously formed NO was ascertained using a combination of the above tools.

Activation of guanylyl cyclase, the target enzyme of NO, results in smooth muscular relaxation in a variety of organs. This type of response may be evoked not only by NO released from the endothelium but also by electrical stimulation of nerves mediating non-adrenergic, non-cholinergic (NANC) transmission signals. Indeed, enhanced production of NO accompanied by smooth muscular relaxation has been observed in several test preparations; complementary confirming evidence is provided

by numerous studies using NO synthase inhibitors and reversal of their actions by L-arginine (see above review papers).

The supposition that NO released from NANC nerves acts as a mediator of "nitrergic" inhibitory transmission (Li and Rand 1991) is strengthened by immunohistochemical evidence of NO synthase localized in enteric neurones supplying small and large guinea-pig intestine (Nichols et al. 1992) or the circular and longitudinal muscle layers of canine proximal colon (Ward et al. 1992), and evidence of an increase in cGMP levels of adjacent ganglion, interstitial and muscle cells that is evoked by field stimulation or exogenous NO (Shuttleworth et al. 1993), and by biochemical identification of NO released by nerve stimulation from myenteric plexus (Wiklund et al. 1993). Finally, Ca^{2+}-dependency of nicotinic and field stimulation-evoked release from NANC nerves of NO determined biologically has been demonstrated on canine ileocolic junction tissue (Boeckxstaens et al. 1993).

However, NO does not appear to be the only mediator of inhibitory NANC transmission, as a substantial body of evidence exists for VIP among other contenders serving a similar function (see reviews by Lundberg and Saria 1987; Furness and Costa 1989; Yau 1989). It is a matter of present research interest to define the relative contribution of either mediator to the effect of NANC stimulation in individual organs. Thus, co-localization of NO synthase with VIP has been demonstrated in rat (Aimi et al. 1993) or guinea-pig intestine (Furness et al. 1992), and in ferret trachea (Dey et al. 1993). In the rat gastric fundus VIP is preferentially released by longer lasting and high-frequency stimulation but NO mainly by shorter trains and at lower frequencies (Boeckxstaens et al. 1992). This is reminiscent of earlier demonstrations of a preferential release of VIP by high-frequency stimulation from cat submandibular (Lundberg et al. 1981) or submaxillary nerves (Andersson et al. 1982), compared to what is known for acetylcholine release. It is likely that both NO and VIP mediate the inhibitory NANC response in guinea-pig and human airways, possibly being differentially released depending on the impulse pattern (Barnes 1992). Also, the effectiveness of NO synthase inhibition to antagonize NANC relaxation varies widely between different organ systems, being large in the rat anococcygeus muscle and low in the gastric fundus (Sneddon and Graham 1992). Since NO and VIP may be acting in series (He and Goyal 1993) both mediators will be considered together.

Up to now only a few studies have been published on the effects of either NO or VIP on acetylcholine release. Belvisi et al. (1991) noticed that NO synthase inhibition facilitated cholinergic neurotransmission

on the guinea-pig trachea (an effect increasing with the frequency of field stimulation), but failed to alter the contractions elicited by exogenous acetylcholine. In a subsequent study α-chymotrypsin, which inactivates VIP, was found to enhance field stimulation-evoked contractions but not those due to exogenous acetylcholine (Belvisi et al. 1993). This led to the conclusion that endogenous NO and VIP may inhibit cholinergic transmission either by postjunctional functional antagonism or via a prejunctional inhibition of acetylcholine release, or both mechanisms. Determination of acetylcholine overflow (loading with [^{3}H]-choline) in guinea-pig trachea (Brave et al. 1991) did not reveal a modulatory effect of endogenous NO at 5 Hz, despite its ability to inhibit contractions evoked by field stimulation at that frequency. Similarly, in isolated tissue from the human trachea and main bronchus Ward et al. (1993) failed to observe any alteration of field stimulation (5 Hz for 2 min) evoked release of [^{3}H]-acetylcholine after inhibition of NO synthase but noticed an enhancement of the contractions. Taken together, these findings show that endogenous NO liberated by electrical field stimulation can inhibit cholinergic neural responses via functional antagonism towards liberated acetylcholine in airway smooth muscle. On the other hand, Sekizawa et al. (1993) reported an enhancement of evoked overflow of acetylcholine determined by the choline oxidase method on rat trachea incubated with N^G-monomethylarginine; in these experiments neostigmine had been added and sample collection was extended for 30 min, leaving open the question as to whether there is a species difference to the above findings or merely the methodology is incomparable.

Although there are some studies suggesting, from indirect evidence, that acetylcholine release is inhibited by exogenous VIP in the airways of both cat and dog (Hakoda and Ito 1990) or endogenous VIP in cat but not in dog (Xie et al. 1991), the situation is complicated: in ferret trachea low concentrations of VIP were found to facilitate cholinergic neurotransmission whereas larger ones caused inhibition (Sekizawa et al. 1988). We are not aware of reports dealing with VIP effects on acetylcholine release determined in the airways.

Likewise, neuromodulatory roles of NO and VIP in the intestine are indicated by some recent observations, but this interesting matter requires further attention. In the guinea-pig taenia coli, NANC relaxation was found to be partly dependent on the integrity of cholinergic pathways and unimpaired synthesis of NO, suggesting that NO exerts its effect prejunctionally by inhibiting the release of acetylcholine (Knudsen and Tøttrup 1992). On the other hand, Middleton et al. (1993) showed that NO has a role in the tonic inhibition of spontaneous

mechanical activity of rat distal colon and assumed that NO serves as an intracellular second messenger for an unidentified inhibitory transmitter rather than providing intercellular communication, or alternatively, that NO is a neurally generated myomodulator. In a direct test using [^{3}H]-choline loading, Kilbinger and Wolf (1994) found that the overflow of acetylcholine from guinea-pig ileum in response to field stimulation is enhanced by NO synthase inhibitors, implying a modulatory role of endogenous NO. This finding contrasts with an observation by Gustafsson et al. (1990) that N^G-monomethylarginine failed to affect field stimulation-evoked acetylcholine release. However, in the latter experiments the drug was added less than 10 min prior to stimulation, which might be insufficient for NO synthase inhibition to be significant.

Two aspects must be considered with regard to a neuromodulatory role of VIP on acetylcholine release in different parts of the gastrointestinal systems: increase of basal and inhibition of evoked release. Enhancement of basal acetylcholine overflow by exogenous VIP has been reported repeatedly (Kusunoki et al. 1986; Yau et al. 1986a; Mayer et al. 1990; Katsoulis et al. 1993). Although VIP is a relaxant of smooth muscle of the circular layer of the gut, it is a stimulant of longitudinal muscle (Yau 1989). Gordon et al. (1990) tested three VIP analogues on guinea-pig ileum for their potency to elicit contractions and to stimulate acetylcholine release that was determined by the choline oxidase method. There was a good correlation between the amounts of acetylcholine released in the presence of a cholinesterase inhibitor, and the magnitude of contraction. These contractions were blocked by atropine but not by tubocurarine, suggesting the VIP effect on cholinergic neurones takes place at a postganglionic site. Dual effects of an agonist on basal and evoked acetylcholine release from the intestine have been observed in several instances, e.g. with muscarinic drugs (Kilbinger and Nafziger 1985), 5-HT (Kilbinger and Pfeuffer-Friederich 1985), GABA (Kleinrok and Kilbinger 1983) and substance P (see Sect. 3.11); the opposing actions were mediated via different receptor subtypes. The latter statement cannot be verified presently in the case of VIP, but field stimulation-evoked [^{3}H]-acetylcholine release was found to be decreased on canine gastric antrum (Mayer et al. 1990) and, to a minor degree, on guinea-pig ileum (Katsoulis et al. 1993). For the role of VIP as an intermediary between NK_1 receptor stimulation, the involvement of a prostanoid, and the inhibitory action of tachykinins on evoked acetylcholine release see Sect. 3.11.

Recent efforts were directed towards elucidating the question of sequential effects and/or interactions of NO and VIP in the intestine,

and to differentiate between several inhibitory mediator candidates. Electrophysiological studies of the effects of various mediators on circular smooth muscle cells of guinea-pig ileum revealed different time courses of inhibitory junction potentials (IJP): A fast IJP due to ATP and a slow one due to VIP (Crist et al. 1992) and to NO (He and Goyal 1993). VIP and NO appear to act in series to produce the slow IJP. It remained open whether VIP is the primary inhibitory transmitter and NO is generated as an intracellular mediator of the postjunctional action of VIP on smooth muscle, or whether NO is the primary transmitter and VIP acting on prejunctional receptors causes release of NO from nerve endings (He and Goyal 1993). Recent evidence from guinea-pig stomach (Grider et al. 1992) or rat colon (Grider 1993) suggests that VIP is the primary transmitter of field stimulation-evoked relaxation. In both preparations VIP stimulates NO production which, in turn, enhances VIP release by a prejunctional action. NO can be generated both from isolated muscle cells by VIP (Grider et al. 1992) and from isolated ganglia of the myenteric plexus by nicotinic stimulation (DMPP) but not by VIP (Grider and Jin 1993). Independent of its origin NO acts to enhance VIP release as a "retrograde modulator" (Grider 1993). However, in the guinea-pig taenia coli NO production is not stimulated by VIP, underlining the notion that the VIP effect appears to be universal whereas the involvement of NO in neurally induced, VIP-mediated relaxation depends on the region of the gut and the presence or relative abundance of NO synthase (Grider et al. 1992).

3.10 Epithelium-Derived Inhibitory Factor

Asthma is a disease in which abundant evidence exists for abnormal neural control of the airways, secondary to the underlying inflammation, but for which there is no satisfactory animal model (see Barnes 1992). An approximation to the sometimes patchy damage of airway epithelium seen in human asthma is provided in animal experiments by mechanical removal of epithelium which has frequently been shown to enhance bronchial responsiveness to various spasmogens (reviewed by Goldie et al. 1990). Most studies were directed at testing the effect of bronchoconstrictor agents before and after shedding the epithelium. The airway epithelium-derived inhibitory factor persists in the presence of cyclooxygenase or lipoxygenase inhibitors (Barnes et al. 1985; Flavahan et al. 1985) , is distinct from NO, and can also be differentiated from platelet-activating factor (Goldie et al. 1990; Skoogh and Ullman 1991).

A prejunctional effect of the epithelium factor was reported by Wessler et al. (1990). Removal of epithelium from isolated guinea-pig trachea resulted in a fivefold increase of basal as well as field stimulation (3 Hz for 2 min) evoked [^{3}H]-acetylcholine overflow (neostigmine and scopolamine present). Since indomethacin enhanced [^{3}H]-acetylcholine overflow from tracheae with intact epithelium by only 89%, a cyclooxygenase product does not appear to account for the main extent of epithelium-derived inhibition of acetylcholine release. Interestingly, Flavahan et al. (1985) had reported an increase in mechanical responsiveness of unrubbed canine bronchial rings to exogenous acetylcholine after pretreatment with indomethacin which the authors ascribed to a partial contribution of a cyclooxygenase product to epithelium-dependent inhibition. The enhanced [^{3}H]-acetylcholine overflow after removal of epithelium was also observed in rat trachea, and it was evident with both preganglionic and transmural electrical stimulation (Wessler et al. 1991). This excludes the possibilities of a ganglionic site of action of the inhibitory factor and of its artificial generation by a strong electrical field. However, the observations of prejunctional modulation of [^{3}H]-acetylcholine overflow in rat and guinea-pig tracheae seem to be restricted to a small transmitter pool labelled by [^{3}H]-choline, because determination of endogenous acetylcholine and its overflow from tracheae of both species did not reveal an inhibitory effect of epithelium on evoked release (Wessler et al. 1993). Hence, it remains to be clarified whether modulation by epithelial factor(s) of acetylcholine release, if at all functionally relevant, is in any way related to the alterations of smooth muscular and particularly bronchoconstrictor responses listed in the above review papers. Although the integrity of the muscular layer of canine bronchi after mechanical rubbing of the epithelial surface has been demonstrated both histologically and by functional tests (Flavahan et al. 1985) the procedures employed by different workers may not only remove epithelium, but also subepithelial parts of the mucous membrane resulting in loss of various tissue components not sufficiently defined yet as possible source of neurotransmitters and/or neuromodulators.

3.11 Tachykinin and Calcitonin Gene-Related Peptide Receptors

Among the tachykinins, substance P has received major attention for possible prejunctional effects since the reports by Holzer and Lembeck (1980) of blockade by tropicamide, an atropine-like drug, and

enhancement by physostigmine, of the tonic phase of substance P-evoked contraction of guinea-pig ileum, suggesting an acetylcholine releasing action of the peptide, and the direct demonstration by Yau and Youther (1982) of [^{3}H]-acetylcholine release. There is now an extensive literature dealing with the postjunctional effects of substance P which is a primary transmitter, e.g. in the enteric or tracheobronchial excitatory muscle motoneurones and contracts all parts of the gastrointestinal or respiratory tract; it is understood that this is due partly to direct actions on the smooth muscle and partly to excitation of cholinergic neurones (for reviews see Barthó and Holzer 1985; Lundberg and Saria 1987; Holzer 1988; Yau 1989; Otsuka and Yoshioka 1993). Increase of basal acetylcholine overflow after substance P has been confirmed for guinea-pig ileum (Fosbraey et al. 1984; Teitelbaum et al. 1984; Tanaka and Taniyama 1985; Vizi and Bartho 1985; Featherstone et al. 1986; Kilbinger et al. 1986; Yau et al. 1986a), rabbit distal colon (Koelbel et al. 1989) and dog gastric antrum (Koelbel et al. 1988b; Mayer et al. 1990). It is noteworthy, however, that the substance P concentration required for acetylcholine release is about two orders of magnitude higher than that causing the rapid contraction of guinea-pig ileum (Fosbraey et al. 1984; Teitelbaum et al. 1984; Kilbinger et al. 1986).

As with VIP, the site of action of substance P and related tachykinins (eledoisin, neurokinins A and B) is the terminal cholinergic neurone, probably the somatic region, because tetrodotoxin abolished acetylcholine release (Yau and Youther 1982; Teitelbaum et al. 1984; Tanaka and Taniyama 1985; Vizi and Bartho 1985; Kilbinger et al. 1986; Koelbel et al. 1989; Mayer et al. 1990; Yau et al. 1992) and hexamethonium did not affect it (Fosbraey et al. 1984; Mayer et al. 1990). Since the order of potencies of four different tachykinins causing contraction of guinea-pig ileum was different from that evoking acetylcholine release, Fosbraey et al. (1984) concluded that these effects were mediated by different tachykinin receptors, namely SP-P receptors (later to be designated as NK_1 receptors; Maggi et al. 1993) for the myogenic and SP-E receptors for the neuronal response. This idea was confirmed when selective antagonists became available, excluding the possibility that potency differences were due to partial agonism of the peptides tested and existence of spare receptors. Thus, Kilbinger et al. (1986) found similar potencies of substance P and eledoisin for longitudinal muscle contraction, effects which were competitively blocked by two substance P antagonists, in agreement with an NK_1 receptor-mediated response. However, eledoisin had a 40- to 80-fold higher potency compared with substance P for eliciting acetylcholine release, and these effects were not inhibited by the sub-

stance P antagonists conforming with an SP-E receptor-mediated response. According to tachykinin receptor terminology introduced subsequently in 1986, acetylcholine release in the guinea-pig ileum is mediated by an NK_3 receptor that is selectively activated by senktide; indirect evidence was provided by Guard and Watson (1987), while acetylcholine release was determined by Fox and Morton (1991) and Yau et al. (1991, 1992).

Another neuronal action of tachykinins has been disclosed by Kilbinger et al. (1986), namely *inhibition* of acetylcholine release evoked by electrical field stimulation. This response does not fade like the direct effect on longitudinal muscle or the spontaneous acetylcholine release, it is not due to an exhaustion of transmitter stores by the preceding facilitatory action, and it is mediated by an NK_1 receptor: the inhibitory potencies of substance P and eledoisin were similar and the effects were blocked by the substance P antagonists at pA_2 values identical to those obtained for muscle contractions. However, an inhibition of the twitch response to electrical stimulation as a possible consequence of reduced acetylcholine release was not observed. An inhibitory action of substance P methyl ester, an NK_1 receptor selective agonist, on acetylcholine release evoked by field stimulation was also observed on canine gastric antrum strips, an effect that was prevented by indomethacin or antiserum to VIP (Mayer et al. 1990). Since exogenous VIP stimulated the formation of PGE_2, and both exogenous VIP and PGE_2 inhibited acetylcholine release, the authors concluded that the inhibitory effect of substance P on acetylcholine release involves sequential VIP and PGE_2 release from the myenteric plexus. It remains to be seen whether an intermediary is involved in NK_1 receptor-mediated inhibition of evoked acetylcholine release in the guinea-pig ileum, too. Recently, the validity of this tachykinin receptor type for inhibition of acetylcholine release has been underlined using selective NK_1 and NK_2 receptor agonists and non-peptide antagonists (Löffler et al. 1994).

There are several studies reporting a facilitatory action on cholinergic neurotransmission by exogenous and endogenous tachykinins in the airways of various species, e.g. guinea pig (Martling et al. 1984; Hall et al. 1989; Stretton et al. 1992; Watson et al. 1993), rabbit (Grunstein et al. 1984; Tanaka and Grunstein 1986) and ferret (Sekizawa et al. 1987). Indirect evidence was provided for acetylcholine release being increased by endogenous substance P acting on postganglionic cholinergic neurones (Sekizawa et al. 1987). More specifically, the facilitatory action has been located at the level of the parasympathetic ganglia where tachykinin(s) released during vagus nerve stimulation activate NK_1

receptors (Watson et al. 1993). Moreover, the latter workers showed that exogenous, but apparently not endogenous, agonists activate facilitatory NK_1 and possibly NK_2 receptors on the postganglionic nerve terminals, whereas NK_3 receptor activation by senktide was ineffective. On rabbit trachea, Inoue et al. (1992) demonstrated enhancements by substance P of both the contraction and [^{3}H]-acetylcholine release evoked by field stimulation which were significantly larger than those observed with neurokinin A, a preferential NK_2 receptor agonist.

A third organ on which prejunctional tachykinin actions were investigated is guinea-pig urinary bladder. Substance P induced a Ca^{2+}-dependent and tetrodotoxin-sensitive release of both [^{3}H]-acetylcholine and [^{3}H]-GABA which were blocked by a substance P antagonist (Shirakawa et al. 1989). Since bicuculline increased substance P induced acetylcholine release, the resulting action of the tachykinin on acetylcholine release appears to consist of a predominant direct facilitatory and a minor indirect inhibitory modulation. The alterations of acetylcholine release were reflected by corresponding changes in mechanical responses of the bladder strips. A role of endogenous tachykinins has also been suggested for the nicotine-induced acetylcholine release from detrusor muscle strips (Shinkai et al. 1991, 1993). Guanethidine or a substance P antagonist reduced evoked acetylcholine release (determined with a cholinesterase inhibitor present) to 30%, and this effect was counteracted by neurokinin A but not substance P methyl ester or senktide, suggesting a tachykinin being released from sympathetic nerves by nicotine and facilitating acetylcholine release via an NK_2 receptor. However, the effect of neurokinin A on basal acetylcholine overflow in the absence of nicotine has not been documented in the latter publications.

Summing up the evidence obtained so far for prejunctional modulation of acetylcholine release by tachykinins, the situation in the gastrointestinal system appears to be established: inhibition of evoked release mediated by NK_1 and increase of basal overflow mediated by NK_3 receptors. In airways and urinary bladder, functional studies suggest the occurrence of facilitatory NK_1 and NK_2 receptors but these proposals will have to be checked finally by determinations of acetylcholine release and employment of selective drugs as tools.

CGRP-mediated modulation of acetylcholine release has only been reported for the intestine. On guinea-pig, porcine and human small intestine, CGRP inhibited the cholinergically mediated twitch responses evoked by transmural stimulation (Barthó et al. 1987; Schwörer et al. 1991). A postjunctional additive relaxant effect as well as an increase in basal tone produced by tenfold larger CGRP concentrations was only

observed on guinea-pig ileum. [^{3}H]-acetylcholine release from this preparation evoked by field stimulation was inhibited by CGRP maximally by 40%, and somewhat larger concentrations of the peptide caused a transient increase of basal [^{3}H]-overflow (Schwörer et al. 1991).

3.12 Opioid Receptors

The regulation of transmitter release by opioid receptors has been the aim of a great many experimental studies which were exhaustively and competently reviewed by Illes (1989). The authors of this article felt unable to present really new additional information to the above topic which covers the time since 1989 and refer to some briefer review papers devoted to special aspects of prejunctional opioid actions on cholinergic neurones (Barnes 1992; Furness et al. 1992).

3.13 Somatostatin Receptors

Somatostatin is contained in enteric nerves (Hökfelt et al. 1975), particularly in noradrenergic postganglionic terminals of guinea-pig (Costa and Furness 1984) or rat (Chevendra and Weaver 1992) mesenteric neurones and is released into the venous effluent of the vascularly perfused guinea-pig ileum after nicotinic stimulation or increase of intraluminal pressure (Donnerer et al. 1984). The peptide has no direct effect on ileal smooth muscle. However, mechanical activity of guinea-pig ileum elicited by electrical field stimulation but not that induced by exogenous acetylcholine was inhibited by somatostatin (Guillemin 1976; Furness and Costa 1979). This suggests a prejunctional modulation of acetylcholine release. There was a pronounced tachyphylaxis of this action which was evident also in electrophysiological observations on cat ciliary ganglion cells where somatostatin reduced the excitatory postsynaptic potential (Katayama and Hirai 1989), or on guinea-pig submucosal neurones where somatostatin produced hyperpolarization and a rapid homologous desensitization (Shen and Surprenant 1993). In the desensitized state, the non-adrenergic inhibitory postsynaptic potential evoked by extrinsic nerve stimulation was abolished thus providing strong evidence for an inhibitory neurotransmitter function of somatostatin on submucosal neurones.

[^{3}H]-Acetylcholine overflow studies have shown that the field stimulation-evoked release from myenteric plexus was inhibited by somatostatin in a concentration dependent manner (Yau et al. 1983a). However,

peptide-induced acetylcholine overflow is differentially affected by somatostatin which decreases both the release and the contractions caused by caerulein or neurotensin (Yau et al. 1983b; Teitelbaum et al. 1984) and acetylcholine release by cholecystokinin octapeptide (CCK-8; Kowal et al. 1989) but fails to modulate these responses of guinea-pig ileum when they are evoked by substance P (Teitelbaum et al. 1984; Yau et al. 1986b; Kowal et al. 1989). By contrast, in canine longitudinal antral muscle, somatostatin enhances basal [^{3}H]-acetylcholine overflow but inhibits acetylcholine release caused by field stimulation as well as that evoked by substance P (Koelbel et al. 1988a). These inhibitory effects are partially mediated by prostaglandins. Since relevant data for guinea-pig stomach or canine ileum are not available, it is not known whether species differences or the special anatomy of the gastrointestinal tract are responsible for the above discrepancies. Interestingly, the endogenous somatostatin content of canine antrum is very low (Koelbel et al. 1988a) compared with guinea-pig ileum. Differences within the alimentary tract are also reflected by immunohistochemical evidence obtained on the rat, in which somatostatin nerve terminals are visible in all parts of the intestine but not in the stomach (Hökfelt et al. 1975). The need to explore the possibility that distinct somatostatin receptors are absent on certain subpopulations of enteric neurones has been emphasized (Yau 1989). However, an alternative to the absence of somatostatin receptors on cholinergic neurones which are excited by substance P is a different intracellular regulation of release caused by the tachykinin compared to that by other peptides, e.g. CCK-8, as pointed out by Kowal et al. (1989). These workers showed that somatostatin inhibited the cAMP-dependent component of CCK-8-mediated acetylcholine release via activation of an inhibitory, pertussis toxin-sensitive, guanine nucleotide-binding protein which inactivates adenylyl cyclase. In contrast, substance P caused acetylcholine release through a mechanism independent of cAMP, probably a Ca^{2+} channel.

Another apparent discrepancy of results may have arisen on methodological grounds rather than species or organ differences. The somatostatin-evoked increase in basal release of acetylcholine is short-lasting and observed only in those studies in which the collection periods of medium were 30 s (Takeda et al. 1989) or 2 min (Koelbel et al. 1988a) whereas an increase was not reported but probably missed when the collection periods were 5 min (Teitelbaum et al. 1984; Yau et al. 1986b).

Thus, somatostatin produces dual effects on cholinergic neurones of gastrointestinal tract, a brief excitatory one and a subsequent inhibitory one, similar to what is known for GABA (Kleinrok and Kilbinger 1983).

In fact, Takeda et al. (1989) have shown that a [^{3}H]-GABA release evoked by somatostatin was paralleled by an increase of electrically evoked twitch contractions of guinea-pig ileum and by acetylcholine release. Both responses were blocked by bicuculline, a $GABA_A$ antagonist. Furthermore, they observed a delayed inhibitory action of somatostatin on both twitches and electrically evoked acetylcholine release which were inhibited by phaclofen, a $GABA_B$ antagonist. From this and some other evidence it was concluded that somatostatin excites GABAergic neurones and that the resulting GABA release mediates the excitatory and part of the inhibitory action on evoked acetylcholine release. It remains to be seen whether VIP is the other intermediary for the latter effect of somatostatin.

No acetylcholine release experiments appear to have been carried out with somatostatin on the airways, but there is functional evidence that the peptide facilitates cholinergic neurotransmission in the ferret trachea, presumably by a prejunctional mechanism, because the contraction elicited by field stimulation but not that caused by exogenous acetylcholine was augmented, and both atropine and a somatostatin antagonist inhibited the effect (Sekizawa et al. 1989).

3.14 Other Neuroactive Peptide Receptors

Histochemical evidence for many peptides is available which occur in nerve cells and their processes, partly together with a classical transmitter in the same neurone (Hökfelt et al. 1980; Furness et al. 1989), and which have been suggested to play a physiological role as primary or cotransmitters, as neuromodulators or as mediators of long-term effects still to be defined; this was discussed with regard to the airways (Barnes 1992), enteric nervous system (Furness et al. 1992) and various peripheral organs (Lundberg and Hökfelt 1986). Compared to the wealth of histochemical data, little is known about the neuronal functions of the majority of peptides. Regarding prejunctional modulation of acetylcholine release, the published work is even scarcer. In the following sections only those peptides will be considered for which at least one transmitter overflow study has been found. Hopefully, gaps of information will be closed in future when selective antagonists become available, and possible receptor functions may be revealed by application of selective agonists although this procedure does not necessarily imitate physiological release of endogenous peptide transmitters.

With regard to the pancreatic polypeptide family, the following observations have been made. Functional studies show an inhibitory

action on cholinergic neurotransmission of NPY released by sympathetic nerve stimulation in dog heart (Potter 1985; Warner and Levy 1989) and of exogenous NPY on blood-perfused dog atria (Ren et al. 1991), on guinea-pig or rat heart (Pardini et al. 1992) and guinea-pig trachea (Stretton and Barnes 1988), respectively. In the guinea-pig colon, a relaxing effect of NPY occurs through inhibition of cholinergically increased tone which is mediated by an NPY-evoked noradrenaline release and paralleled by the inhibition of [^{3}H]-acetylcholine overflow evoked by a high K^+ concentration (Wiley and Owyang 1987). High K^+-evoked [^{3}H]-acetylcholine release from rat nodose ganglia is also decreased by NPY, through a pertussis toxin-sensitive mechanism (Wiley et al. 1990). Similarly, the NPY-related peptide YY (PYY) decreases K^+-evoked [^{3}H]-acetylcholine release by inhibiting adenylyl cyclase rather than acting distally to cAMP formation, and relaxed guinea-pig stomach longitudinal muscle (Wiley et al. 1991). Both NPY and PYY, as well as bovine pancreatic polypeptide (bPP), have been shown to inhibit fast excitatory postsynaptic potentials in guinea-pig gastric myenteric neurones, indicative of a presynaptic modulation of acetylcholine release (Schemann and Tamura 1992). On rat pancreatic slices, rat pancreatic polypeptide appears to diminish both K^+-evoked amylase and [^{3}H]-acetylcholine release (Jung et al. 1987). Another pancreatic enzyme secretion regulatory peptide is pancreastatin, which has been reported to inhibit cholinergically induced pancreatic secretion in the rat in vivo and to decrease K^+-evoked amylase and [^{3}H]-acetylcholine release in isolated lobules (Herzig et al. 1992).

The following peptides have all been shown to cause a transient increase of the basal overflow of [^{3}H]-acetylcholine from guinea-pig ileum, in a Ca^{2+} dependent and tetrodotoxin-sensitive manner, this effect usually being regarded responsible for longitudinal smooth muscular contraction: endothelin (Wiklund et al. 1989c), neurotensin (Teitelbaum et al. 1984; Yau et al. 1983b; Nakamoto et al. 1987; Rakovska 1993), CCK-8 and caerulein, a CCK-like peptide (Teitelbaum et al. 1984; Yau et al. 1983c; Kowal et al. 1989). A receptor classification was carried out by Lucaites et al. (1991) who used selective antagonists and found that the CCK_A receptor mediates both substance P and acetylcholine release, and that the CCK_B receptor is responsible for acetylcholine release only. Leu^{13}- and Met^{13}- (porcine) motilin released [^{3}H]-acetylcholine from rabbit duodenum and caused a contraction, the tonic component of which appeared to be due to endogenous acetylcholine (Kitazawa et al. 1993). However, as observed with substance P (see Sect. 3.11), the EC_{50} for [^{3}H]-acetylcholine release was 100 times that for

eliciting the contraction response. Only for endothelin has a small inhibition of [^{3}H]-acetylcholine release and twitches evoked by 3 Hz transmural stimulation of guinea-pig ileum been reported (Wiklund et al. 1989c). Using various agonists and selective endothelin receptor antagonists Warner et al. (1993) characterized an ET_A receptor mediating the contraction and an ET_B receptor depressing the twitches evoked by transmural stimulation of guinea-pig ileum.

Galanin, which is not a member of a known peptide family, is stored, for example, in cardiac postganglionic nerves and is responsible for inhibition of vagally induced bradycardia by sympathetic nerve stimulation (in the presence of β-adrenoceptor blockade) in the anaesthetized cat, in which NPY as mediator of this action can be excluded (Revington et al. 1990; Ulman et al. 1993). In contrast to this, in the dog, where exogenous or endogenous NPY released from sympathetic nerves decreased bradycardia after vagus nerve stimulation (see above), injected galanin was ineffective (Ulman et al. 1993), and galantide, a putative galanin antagonist, did not affect vagal attenuation produced by sympathetic nerve stimulation, whereas it did in the cat (Ulman et al. 1993). Measurements of [^{3}H]-acetylcholine release were carried out on cultured guinea-pig myenteric plexus neurones (Mulholland et al. 1992). Galanin inhibited the acetylcholine release evoked by high K^+, substance P, CGRP, VIP and CCK-8, and in all cases inhibitions were reverted by pretreatment of neurone cultures with pertussis toxin. Galanin also antagonized contractions of guinea-pig taenia coli muscle strips exposed to CCK-8 or CGRP, but did not affect those produced by substance P, in line with a predominantly direct effect of this peptide on smooth muscle. Field stimulation-evoked [^{3}H]-acetylcholine release from guinea-pig small intestine myenteric plexus was potently decreased by galanin (Yau et al. 1986c), and that evoked by 5-HT from submucosal plexus was differentially affected, namely decreased by a submicromolar and increased by a micromolar concentration (Yau et al. 1990). This and some other evidence suggests that cholinergic neurones of the two tissues react essentially in a dissimilar fashion.

4 Concluding Remarks

Within the last decade much work has been devoted to the prejunctional modulation of neurotransmitter release from the peripheral autonomic nervous system. In some areas of this field only little progress has been

made. It is still unclear where prejunctional receptors are located, either directly at the varicosity from which transmitter is released or distant from the site of release. The latter possibility would involve lateral inhibition or inhibition of propagation of action potentials. It is still unclear whether intermittency of transmitter release (i.e. failure of action potentials propagated up to a varicosity to induce exocytosis with high probability) is a universal phenomenon or is observed in a few models only, and whether or not heteroreceptors act by enhancing the intermittency (see Stjärne 1989, Stjärne et al. 1990). Furthermore, the mechanism of the dependency on calcium ions has not been satisfactorily resolved. It is still an open question whether prejunctional heteroreceptors inhibit the influx of calcium ions directly by blockade of neuronal calcium channels or indirectly via activation of potassium channels. Little is known about second messenger systems involved in prejunctional modulation via heteroreceptors on peripheral autonomic nerves.

On the other hand, considerable progress has been made in other areas. It is clear now that cotransmission is a widespread phenomenon, and release of the cotransmitter is subject to modulation by heteroreceptors, though not necessarily to the same extent as through the identical receptor for the classical neurotransmitter. Yet the fact of concomitant prejunctional modulation of release of both classical transmitter and presumptive cotransmitter has strengthened the case for their origin from the same type of nerve fibre. The greatest development was observed in the field of receptor classification, as can be seen in all of the tables presented here. This is true for peptide and non-peptide receptors.

Nevertheless, tissue and species differences in prejunctional receptor supply and diversity of prejunctional responses mediated by even one and the same subtype of receptor make it difficult to predict the functional role of prejunctional modulation in a given model exerted by the endogenous agonist(s). For that reason it is impracticable now to design *general* schemes for receptor equipment of autonomic neurones and direction of qualitative changes in transmitter release, increase or decrease, respectively. The great spectrum of possible responses is further underlined by experimental proof of well-defined prejunctional receptors when physiological agonists are applied at "pharmacological" doses. However, there is much less proof that the concentrations of such agonists attained at the prejunctional receptors under physiological or pathological conditions are sufficiently large to cause a response similar to that obtained after external application. In other words, despite much evidence for the occurrence of various prejunctional receptor types obtained with exogenous agonists and in vitro experiments, and with

transmitter release usually evoked by electrical field stimulation, few modulatory mechanisms have withstood the test that they can be reproduced by orthodromic stimulation of the nerve supply of the organ under study. Thus, a wide field in this area is open to further research efforts.

For example, agonists at muscarinic receptors are generally found to cause inhibition of evoked noradrenaline release in rat, mouse, guinea-pig, rabbit and dog heart (14 references in Table 1), or chicken and cat heart (see Muscholl 1980). These findings can be duplicated by release of the endogenous agonist acetylcholine through electrical vagus nerve stimulation in those species that were tested (rat, rabbit, dog: six references in Table 1), and there was no negative evidence. As a matter of fact, however, closer inspection revealed a facilitation of noradrenaline release as well, which is mediated by the M_1 receptor, is somewhat delayed and weaker than the inhibition mediated by the M_2 receptor (Fig. 1,

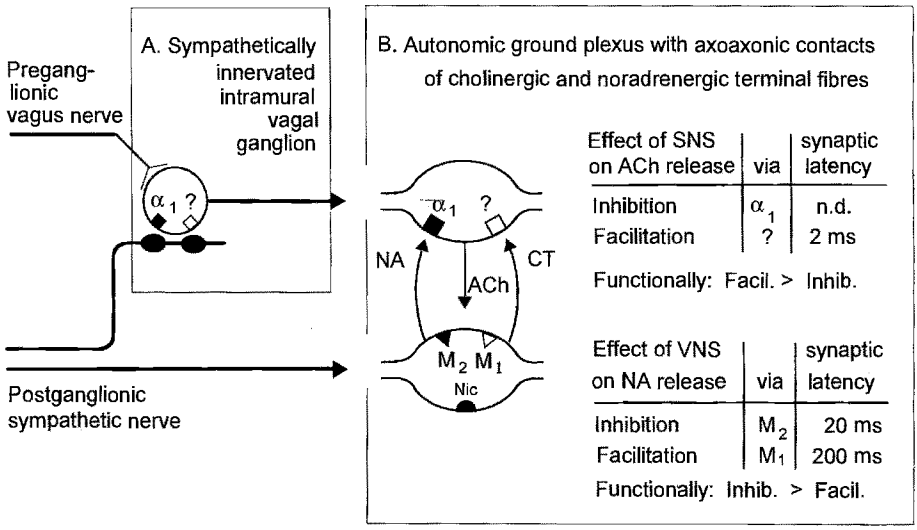

Fig. 1A,B. Activation of mutual prejunctional heteroreceptors by sympathetic and vagus nerve activity in the rabbit perfused atria preparation, as defined by transmitter overflow measurements. (Based on results of Muscholl and Muth 1982; Habermeier-Muth and Muscholl 1988; Habermeier-Muth et al. 1990; Muscholl and Habermeier-Muth et al. 1991a,b.) Presently it cannot be decided which anatomical arrangements (**A** or **B**, or both) are functionally valid for modulation of acetylcholine (*ACh*) release caused by noradrenaline (*NA*) and by an unknown cotransmitter (*CT*) released by sympathetic nerve stimulation (*SNS*). For modulation of NA release that is solely due to ACh, **B** is valid. The nicotinic receptor at noradrenergic terminal fibres (*NIC*) is not activated by ACh released by vagus nerve stimulation (*VNS*). n.d., not determined. In addition to α_1-receptor-mediated inhibition, and CT mediated facilitation, of ACh release evoked by VNS, a minor adenosine A_2 receptor-mediated facilitation of ACh release during SNS has been suggested (Muscholl and Habermeier-Muth 1992). In dog heart, in vivo inhibition of cholinergic transmission through NPY released by SNS has been demonstrated (Potter 1985; Warner and Levy 1989)

see also Sect. 2.1). Two papers confirm M_2 receptor-mediated inhibition of noradrenaline release in rat stomach and rabbit jejunum after vagus nerve stimulation, but regarding other organ systems the evidence is scanty. Stimulation-evoked acetylcholine release is modulated by exogenous adrenoceptor or dopamine receptor agonists in several test preparations (Tables 7–9), mostly inhibited via α_2-, β_1- and β_2- or D_2 receptors. Stimulation of sympathetic nerves causes α_2-adrenoceptor-mediated inhibition of acetylcholine release in guinea-pig and rabbit intestine (six references in Table 7). However, a general scheme cannot be established since firm evidence exists for α_1-receptor-mediated inhibition of acetylcholine release in rat heart caused by exogenous agonists (Table 7). In rabbit atria, sympathetic stimulation causes α_1-receptor-mediated inhibition of acetylcholine release that is overridden by a facilitation due to release from noradrenergic nerve fibres of an unknown cotransmitter (Fig. 1). Although the arrangement of the latter figure is confined to a single species, organ and experimental condition it may be factitious with respect to physiological impulse patterns still, but may serve a model function for analysis of pulse-to-pulse effects at axoaxonix synapses in terms of transmitter release and functional consequences at heteroreceptors with a high time resolution. This model may also be useful for interpretating the synaptic events occurring in the central nervous system.

Quite a few agonists and antagonists selective for subtypes of many receptor classes are available now. Analysis of responses with respect to receptor heterogeneity or functional diversity has been the goal of many studies carried out in recent years. Development of prejunctional agonists is still a promising approach to selectively influence autonomic nerve function, and thus organ responses. Likewise, selective antagonists with preferential prejunctional activity might be useful tools to assess further the physiological or pathophysiological role of prejunctional heteroreceptors and to affect organ function via a prejunctional mechanism of action.

Acknowledgements. We thank Prof. H. Kilbinger for many helpful suggestions and stimulating discussions. Experimental work of the authors mentioned in the review was supported by grants from the Deutsche Forschungsgemeinschaft.

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Editor-in-charge: Professor E. HABERMANN

Subject Index

A-band 37
acetylcholine overflow, epithelium factor 369
– –, trachea 339, 340
– release 364 ff
– –, α_1-adrenoceptor 328, 329, 339
– –, β-adrenoceptor 340, 342, 343
– –, 5-HT receptors 341, 347, 348
– –, adrenergic inhibition 266
– –, adrenoceptors 329–340
– –, amino acid receptors 360–364
– –, calcitonin gene-related peptide receptors 372, 373
– –, CGRP modulation 372, 373
– –, choline oxidase method 367
– –, dopamine receptor agonists 380
– –, – receptors 341, 344–346
– –, field stimulation 327, 328, 371
– –, high K^+ 328
– –, histamine receptors 349
– –, intestinal 339
– –, neuroactive peptide 375–377
– –, opioid receptors 373
– –, prostanoids and leukotrienes 351, 358, 359
– –, purinoceptors 350, 351, 352 ff
– –, somatostatin 373–375
– –, tachykinins 369–372
– –, VIP 365, 366, 367, 368
ACTH, noradrenaline release 326
actin, ATP binding site 91, 93, 95
– binding site 3
–, Ca^{2+} binding 33
–, cross-bridge dissociation 45
–, inactivation 23
adenine moiety 95, 96
adenosine 92, 93
–, agonists and antagonists 352
–, cholinergic transmission 350, 352 ff
–, prejunctional effects 350
–, receptor subtypes 297, 298, 300 ff
– receptors/purinoceptors 297
– segment 93, 94
–, sympathetic transmission 300 ff
adenylate kinase, development 143
ADP dissociation 13, 15, 16
adrenaline, acetylcholine release 340
–, noradrenaline release 285
α-adrenergic agonists and antagonists 330 ff
adrenergic-cholinergic interaction 379
α-adrenoceptor, acetylcholine release 329–340
–, cholinergic transmission 330 ff
α_2-adrenoceptors, prejunctional 285, 296
β-adrenoceptors 285
–, cholinergic transmission 340, 342, 343
–, prejunctional 340
–, sympathetic transmission 285, 286
aldose reductase, lens 146
alkali light chains 46, 47
amino acid receptors 305
angiotensin II 286
–, β-adrenoceptor stimulation 315
–, cotransmitter release 315
– levels 317
–, prostanoid formation 315, 316
–, receptors 314
–, sympathetic transmission 314 ff
angiotensins, cholinergic interaction 316
anion channel, voltage-dependent 124, 126
antiinflammatory drugs, non-steroidal 358

apocytochrome c 233
arachidonic acid metabolites 299
asthma 368
ATP analogs 95, 96
– binding site, hexokinase 90 ff
– – sites, amino acid sequences 93, 94
–, caged 21
– hydrolysis 216, 234, 235, 242
–, insulin synthesis, regulation 166
–, interaction distance 19
–, mitochondrial, hexokinase 133, 134, 135
– overflow 315
–, prejunctional, acetylcholine release 350
–, preprotein translocation 217, 218, 233
– requirement, translocation 233, 236, 238
–, synaptic 303, 368
–, translocation process 233, 234
–, translocator protein 238
ATP-hexokinase complex 90
ATPase, actin-activated 47
atrial natriuretic factor 313
– – –, sympathetic transmission 313, 314
autoreceptors 266

baclofen 361
binding protein, immunoglobulin 203, 235 ff
– proteins, NLS 223, 224
– –, presequence 219, 222
biotin 162
bovine pancreatic inhibitor 241
bradykinin, noradrenaline release 304, 325
brain, glucose utilization 146, 147
–, ketone bodies 146
bronchial responsiveness, spasmogens 368
bronchoconstriction, histaminergic modulation 349

C-protein 3, 10
Ca^{2+} binding 38
– –, troponin 2, 3
– –, troponin C 48
–, caged 21
– calmodulin, hsp90 208
– channels 166, 312, 351, 362, 363, 372, 374, 376, 378
– –, N-type 319
– current 280, 281, 305, 327
–, mechanical V_{max} 26, 29
–, muscle shortening 13–15
–, muscle tension 6, 7, 32 ff, 36
–, myofilament sensitivity 7, 8, 10, 33, 35, 36
calcitonin gene-related peptide, noradrenaline release 319, 324
calnexin 240
capsaicin 319
captopril 317, 318
carmoxirole 283
β-cell tumor cells 169, 170
β-cells, pancreatic, cultured 167
chaperone proteins, cytosolic 218
chaperones see also hsp
–, molecular, definition 200
–, –, eukaryotic cells 202
–, targeting mechanisms 219–222
chaperonins, definition 205
–, function 206, 207
chemical cage, photolysis 21
chloramphenicol acetyltransferase gene 165
chloroplast, membranes 234
–, protein folding 243
–, protein import 234, 235
–, stromal factors 234, 235
cholecystokinin 374
– receptors, acetylcholine release 376
cholinergic agonists 268 ff, 281, 282
– ganglia 327
– mechanosensory transmission, GABA 362
cimetidine 291, 292, 293
citrate synthase 209
co-chaperonins 205, 207
cocaine 339
connectin 37
cotransmitter, release 378
cotransmitters 375
cross-bridge detachment 13, 14, 15, 16, 18
– interaction 32, 33
– – cycle 5, 10, 16, 18, 19, 20, 21, 24, 33
– model 23
–, redistribution 22
– transition, kinetics 20, 21, 22
–, working distance 20
cross-bridges, strongly bound 7, 8, 10, 11, 34
cyclophilin 206, 210, 211, 224, 241, 243
cyclosporin A 210, 211, 241
cystic fibrosis transmembrane conductance regulator 239

cytochrome DHFR fusion proteins 227, 232
– oxidase, development 143
– –, fusion protein 226

DCCD 124, 125
diabetes, gestational 172
– type 2, glucokinase 171
differential scanning calorimetry 90
dihydrofolate reductase 226, 227
dioxin receptor 208
DnaJ homologs 215–217, 219
– system 202–205
DnaK system 202–205
dopamine receptors 283
– –, acetylcholine release 341, 344–346
– –, prejunctional 283, 284
– release 284
–, sympathetic transmission 284
DPCPX 298 ff, 303

eledoisin 370, 371
endoplasmic reticulum, lumen, function 239
– –, membrane, import apparatus 221
– –, preproteins 220
– –, protein folding 239
– –, – mutation 239, 240
– –, – translocation 235 ff
– –, target membrane 220, 221
endothelial factors, sympathetic transmission 311–313
endothelin, acetylcholine release 377
– receptor 313
enzyme, centrifugal fractionation 122
epithelium-derived inhibitory factor, airway 368, 369
estrogen receptor complex 224
excitation-contraction coupling 22
– –, failure 7

farnesylation 215, 216
field stimulation 281
FK binding proteins 210, 211
flux ratio experiments 119
force-velocity data 12, 14, 16, 49, 50, 51
fructose 1-phosphate, glucokinase 159
– phosphorylation 155
– transport 139
furosemide 362, 363
fusion protein 226, 227

G protein 280, 291, 314, 317, 327
GABA, chloride conductance 362, 363
–, cholinergic interactions 360–363, 367, 375
–, endogenous, release by neuropeptides 361, 362, 375
– receptors, prejunctional 305
– –, sympathetic transmission 310
$GABA_A$ receptor, peripheral 362
$GABA_B$ receptor, central 360
– – peripheral 360–363
– –, subclassification 310
GABAergic tone, retina 360, 361
galactokinase family 68
galanin, acetylcholine release 377
–, noradrenaline release 326
gene duplication-fusion concept 70, 71, 78, 87, 95
general insertion pore 227
glia, glucose transporters 145
γ-gliadin 212
glucagon, glucokinase gene 162
glucocorticoid receptor, translocation, nuclear 224
glucokinase 69, 72, 105, 108, 113, 118, 119, 121, 138, 140
– activity, regulation 169
–, ambiquitous behavior 161
–, amino acid sequence 168
–, anterior pituitary cells 169, 170
–, expression 168, 169, 170
– gene, type 2 diabetes 171
–, glucose sensor 164, 165
–, hepatic 158 ff
–, –, distribution 163
–, –, glucose phosphorylation 158
– –, intracellular location 161
– locus, mutations 171, 172
– mRNA 163, 164
– –, insulin 161, 162, 163
– –, variant 170
–, pancreatic 164 ff
–, –, glucose phosphorylation 164, 165
–, –, mRNA 168
–, regulatory protein 160
glucokinases, definition 66
glucose 6-phosphate 116
– –, binding site 117
– – – –, hexokinase 100–102, 106
– –, inhibition 100, 103, 160
– binding site, hexokinases 98–100, 101
– metabolism, hepatic 158, 159
– paradox 158
– phosphorylation, insulin secretion 164, 165
– repression phenomenon 121

glucose 6-phosphate (*Contd.*)
– sensor 163, 164, 165
– transport, endoplasmic reticulum 139
– transporter 139, 140, 144, 145, 148, 164, 165, 167, 169
α-glucosidase 209
glutamate receptors, acetylcholine release 363, 364
– –, sympathetic transmission 310
glycerol kinase, ATP binding 94
glycogen, synthesis 159
glycolytic enzymes 143, 147
–/oxidative interaction 133, 134, 137
GroEL system 206, 207
GroES 207
GrpE system 202–205, 216
guanidine derivatives 296, 297
guanylyl cyclase, noradrenaline release 312

heat shock proteins see hsp
hemagglutinin 239, 240
–, protein assembly 241
heme, translocation 232
heteroceptors see also receptors
–, definition 266
–, noradrenergic-cholinergic interaction 379
hexamethonium 283
hexokinase(s), 38 kDa 77
–, 50-kDa 69, 70, 73, 75, 76, 78
–, 50-kDa 84, 86, 105
–, 100-kDa 84, 86, 105
–, 100-kDa, evolution 69, 70, 75, 76, 84 ff
–, 100-kDa, mammalian, function 78 ff
–, actin 139
– activity, chronic stimulation 147, 148
– –, perturbation Y, metabolic 147
– –, tumor cells 149, 150, 152
–, ambiquitous behavior 137
–, amino acid sequence 71 ff
–, Arrhenius plot 155
–, astrocytes 122
–, ATP binding 75, 112, 114, 115, 119
–, – – site 80, 83, 85, 86, 90 ff
–, bilobar structure 89
– binding, Mg^{2+} 126
– –, microsomal 128, 130
– –, mitochondrial 122 ff, 155, 157
– –, –, functional significance 132–136, 137
– –, nonmitochondrial 127, 128
– – protein 123, 126
–, brain 122, 130, 142 ff
–, C-terminal half 83, 84, 85, 86, 87, 90, 101, 103, 105, 160
–, cDNA cloning 71
–, changes in maturation 156, 157
–, chymotrypsin treatment 124, 125, 131
–, conformational change 98, 99, 102
–, Cys residues 105, 106
–, cytoplasmic 155
–, defect, Alzheimer's disease 151
–, deficiency 151
–, definition 66
–, degradation 157
– distribution, intracellular, activity regulation 136–138
– –, –, alcohol 137
– –, –, ischemia 137
–, disulfide bridges 107, 108
–, elution 130, 131
–, erythrocytes 151, 156
–, evolution 67–78
–, expression, tumor cells 150
– family 67, 75
–, glucose 1,6-biphosphate 142
–, – 6-phosphate binding site 83, 84, 85, 100 ff
– glucose 6-phosphate inhibition 77, 78, 82, 83–87, 100, 103, 105, 116, 117, 136, 137, 142, 152, 157, 160, 166, 173
–, – 6-phosphate-induced release 130, 131, 138
–, – binding 112
–, – – site 80, 83, 84, 85, 86, 89, 98 ff
–, – transport 121, 138–140
– glucose-induced interaction 97, 102
–, guinea pig 143
–, human placenta 155, 156
–, immunofluorescence 143, 146
–, immunohistochemical localization 122, 123, 130
–, inhibition, competitive 115, 116
–, inorganic phosphate 103, 104, 142
–, intracellular distribution 121 ff
– isozymes 69, 70
– –, 100-kDa 173
– –, genes 150, 151, 152
– –, physiological role 120, 121
–, kinetics 111
–, lens 145, 146
– levels, development 142 ff
– –, relation to energy demands 147, 148
–, limited proteolysis 157

–, mammalian, activity, regulation 141 ff
–, –, physiology 120 ff
–, Mg^{2+}-ATP 98
–, mitochondrial, tumor cells 135, 136
–, mnemonic model 119, 120
–, multiple forms of isozymes 154
–, muscle 143, 145, 147
–, mutagenesis experiments 81
–, N-terminal half 84, 85, 86, 87, 90, 107, 150
–, – segment 125
–, particulate 142, 143, 149
–, phosphorylation 108, 109, 110
–, P_i insensitive 103, 152, 157
–, plasma membrane 138, 139
–, protein kinase activity 108, 109
–, random addition mechanism 114
–, regulatory site 82
–, reticulocytes 156, 157
–, sequential mechanism 111
–, slow transition model 119, 120
–, sperm membrane 110
–, starvation 153, 160
–, steric obstructions 84
–, structure 88 ff
–, substrate binding site 67, 71, 75, 90 ff
–, – inhibition 142
–, sulfhydryl groups 104–106, 107, 153, 154
–, tetrameric status 127
–, trehalose 6-phosphate 70
–, type I and II isozymes 80
– type I, catalytic function 80–82
– –, crystallized 173
– –, gene mutation 150, 151
– – isozyme 122, 154, 155
– –, mRNA 144, 145
– –, – TSH 145
– –, N-terminal half 101, 103
– –, regulatory function 82
– –, structure 79
– type I–III, kinetic studies 113 ff
– type II and type III isozymes, functional organization 87, 88
– type II, glucose transporter 148
– –, insulin 140, 149, 150, 153, 154
– – isozyme 87, 103, 104, 105, 131
– –, mRNA 148
– –, multiple forms 153, 154
– –, muscle 147
– –, – denervation 148, 149
– –, – inactivation 149
– –, myopathies 152
– type III, glucose 6-phosphate 142
– – isozyme 104, 105, 132
– –, nuclear periphery 141
– type IV, gene mutation 158
– –, genes 161, 162
– –, isozyme 85, 105
– –, kinetics 118, 119, 120
– –, mammalian 158 ff
– wheat germ 76, 77
–, yeast 69, 88, 89
–, – and mammalian 71
–, –, kinetic studies 111, 112
hexokinase-mitochondrial interaction 133, 136, 137
histamine, endogenous 291
–, receptor agonists 291 ff
–, – subtypes 291, 292, 293, 294, 295
– receptors 290
– –, cholinergic transmission 349
–, sympathetic transmission 291
homoserine kinase 68
hsp, ATP binding 91, 92, 93, 95
–, low molecular weight 209
hsp60 234
–, protein folding 242, 243
–, system 213
hsp70 202, 203, 204, 205, 206, 216, 218, 219, 222, 223
–, ATP requirement 233
–, chloroplasts 234, 235
–, ER translocation 235–238
–, function 212 ff, 216
–, mitochondrial, mutations 230, 231, 232
–, presequences 219
–, protein binding 230
–, translocation machinery 242
–, translocation mechanism 227 ff
hsp90 239
–, family 213
–, function 208
–, nuclear import 223, 224
hsp104 200, 209
hsp110 family 213
hydrophobic collaps 205
5-hydroxytryptamine, endogenous 290
– receptor subtypes 286 ff
–, receptors 286
– –, acetylcholine release 341, 347, 348
–, –, classification 347, 348
–, –, localization 348
–, sympathetic transmission 290

imidazoline receptor, prejunctional 296, 297
– receptors 296
immunoglobulins 239, 240
immunophilins 210, 223, 224
import channel 221
impromidine 349
indomethacin 304, 351, 369
inorganic phosphate, ATP competition 103, 104
– –, binding, hexokinase 103, 104, 106, 142
insulin, glucokinase 161, 162, 164 ff
– promoter 165, 166
– secretion, $AtT20_{ins}$ 170
– –, regulation 164, 165, 166, 167

junction potentials, inhibitory 368

K^+ channels, ADP-activated 166
– –, ATP-sensitive 166
kainate receptor 364
KAR2 gene 235, 236
ketanserin 287, 288, 289, 290
α-ketoglutarate dehydrogenase, Alzheimer 151
ketohexokinase 68
kinetics, hexokinases 111 ff
kynurenic acid 364

α-lactalbumin 215
lactate formation, avoidance 134
laser diffraction pattern 22
leukotriene receptors, sympathetic transmission 299, 306 ff
leukotrienes 358
light chain, alkali 30–32
– –, regulatory 30
light-harvesting chlorophyll-binding protein 234
losartan 316, 317

major histocompatibility complex 203
mercaptoethanol 153
metabolism, hexokinase activity, regulation 141 ff
methacholine 268, 269, 270, 271, 273, 282
methiothepin 287, 288, 289, 290, 347
methotrexate, unfolding reaction 226, 232
microsomes, PDI 240
–, protein translocation 237
mitochondria, ATP-depleted 233
–, contact sites 123
–, import stimulating factor 222
–, membrane potential 229, 231
–, protein folding machinery 227, 242
–, protein translocation 225 ff
monoamine oxidase, mitochondrial marker 127, 129, 130
morphine, acetylcholine release 347
muscarinic receptors 267
– –, axonal transport 267
– –, heterogeneities 279, 280
muscimol 361, 362
muscle, contractile properties 3, 4
– contraction, myosin light chains 30–32
– –, protein isoforms 3, 4
–, cross-bridge interaction 3, 5, 7, see also cross-bridge
–, development 4
– fatigue 7
– fiber, fast/slow 4, 7, 17, 21, 25, 26 ff, 34, 36, 40, 42, 45, 48, 50
– –, fatigue resistance 9
– –, mechanics, assessment 4 ff
– –, shortening velocity 26–29
– –, skinned 6, 21, 22, 35, 36, 49, 51
– –, tension 5–10
– –, type I and II 143
–, force recovery 18, 19, 21 ff
–, – transient, photorelease 21
–, force-velocity relation 12, 14, 16, 49, 50, 51
–, isometric tension 5–10
–, length reduction 18, 22
–, lengthening 12
–, regulatory proteins 3, 32 ff
–, relaxation 38–40
–, resting tension 38
–, shortening velocity 11–17, 44, 49 ff
–, stiffness 10, 11
–, stretch, titin 37
– tension, Ca^{2+} sensitivity 34–36, 45
– –, cooperative activation 34
– – transients 18–24, 25
– twitch 6, 7, 17
– unweighting 44
–, V_{max} 12 ff, 44 ff, 26, 27, 31
– work 16, 17
myenteric plexus 361, 368, 373
– –, acetylcholine release 328, 341, 347, 349
– –, NO release 365
myofibril isoforms, experimental influences 41 ff
– –, natural transition 24 ff
myosin, accessory proteins 10

–, ATP interaction distance 19, 20
–, – activity 26
–, fast and slow isoforms 49 ff
– heavy chain, expression, plasticity 41
– – –, isoforms 24 ff, 41
– – –, twitch kinetics 25, 26–29
– isoform expression, mechanical activity 43–45
– – –, neural activity 42, 43
– – –, thyroid hormone 43
– isoforms 4, 24, 26
– –, ADP release 13
– –, relative effects 49 ff
– light chain 45, 46, 47
– – –, isoforms 24, 42
– – –, phosphorylation 9
–, multiple isoforms, expression 49, 50, 51
–, slow 25
–, subfragment 1, 24
–, subunit structure 24, 25
–, thick filaments 2, 3, 16

N-ethylmaleimide 8, 34, 214, 280
NADPH-cytochrome *C* reductase 128, 129
NEM 24
neurokinin A 324
neuromuscular interval 266
neuropeptide Y 285
–, cholinergic transmission 376, 377
–, cotransmitter 318
–, receptor agonists 320 ff
–, receptors 318
–, sympathetic transmission 319 ff
neurotensin, noradrenaline release 327
nicotinic receptors 282
NinaA 211
nitric oxide, acetylcholine release 364 ff
– –, endogenous, field stimulation 366
– –, endothelin receptors and epithelial factors 311
– –, myomodulator 367
– –, retrograde modulator 368
– –, sympathetic transmission 311, 312
– –, synthase 364, 365, 366
– –, –, VIP colocalization 365
– –, transmitter 312, 364, 365
– –, VIP interaction 368
non-adrenergic, non-cholinergic transmission 364, 365, 366
noradrenaline release 318, 319
– –, adenosine receptors 297, 298 ff, 303, 304, 310, 351
– –, β-adrenoceptors 285, 286
– –, amino acid receptors 305, 310
– –, angiotensin receptors 314 ff
– –, atrial natriuretic factor 313, 314
– –, cholinergic modulation, physiological 379
– –, dopamine receptors 283
– –, EDRF 311–313
– –, histamine receptors 290–296
– –, (5-HT) receptors 286, 290
– –, hypoxia 297, 303
– –, imidazoline receptors 296, 297
– –, ischaemia 282
– –, muscarinic facilitation 280, 379
– –, – heteroreceptors 267–282, 283, 379
– –, – inhibition 266, 267 ff
– –, nicotinic receptors 282, 283
– –, opioid receptors 324, 325
– –, ouabain 282
– –, polypeptides 325, 326, 327
– –, prostanoids 304, 306 ff
– –, sensory neurotransmitter 319, 324
– reuptake, insulin 327
– uptake 1 carrier 272, 291
nuclear localization sequences 223, 224
– pore complex 222, 223
nucleus, protein import 222–225

ondansetron 347
opioid actions, cholinergic nerves 325
– receptor interaction 325
– –, sympathetic transmission 324, 325
– receptors 324
– –, acetylcholine release 373
organelles, biogenesis 200, 201
oxidative phosphorylation, hexokinase, mitochondrial 133, 134, 135, 137
oxotremorine 268, 270, 271, 272, 274, 279, 341

parvalbumin, comparative aspects 40
–, Mg^{2+} 38, 39
–, muscle damage 41
–, – relaxation 38, 39, 40
pattern recognition approach 91, 93
peptides, acetylcholine release 375 ff
–, cotransmitters 375
peptidyl-prolyl isomerases 209–211
pH, muscle tension 8, 9
phaclofen 361
phosphate 2 segment 93, 94, 95, 97, 98
–, inorganic 5, 6, 9, 20, 21, 23, 83, 86, 103
–, –, glucose 6-phosphate inhibition 103, 104, 152

phosphoglycerate kinase, mRNA 144
pirenzepine 268 ff, 279
plastocyanin 234
polypeptide, extended conformation 226, 227
polyphosphate, substrate 68, 69
porin 218
–, mitochondrial 124, 125
power/load curves 16, 17
precursor proteins, translocation 212 ff, 217, 218
preprotein synthesis 201
preproteins, chloroplast, binding and import 217, 218
–, cytosolic 215
–, –, translocation-competence 217
–, import into organelles 217, 218, 219
–, low molecular weight 222
–, organelles translocation 225 ff
–, peroxisomes 217, 218
–, translocation, conformation 225, 226, 227
–, unfolding reaction 226
presequence binding factors 219, 222
presequences, translocation 229, 233
procollagen 212
proline, conformers 209
prolyl 4-hydroxylase 212
– isomerization 210
prostacyclins, acetylcholine release 359
prostaglandins, acetylcholine overflow 351, 358, 359
–, postjunctional role 358, 359
–, prostanoid receptor, classification 305
–, synergismen with VIP 371
prostanoid and leukotriene receptors 304
– receptors, sympathetic transmission 305, 306 ff
prostanoids, endogenous 359
protein disulfide isomerases 200, 211, 212
– expression, myofibrillar 4
– folding, chloroplasts 243
– –, ER 239–242
– – machinery 227, 242
– – mitochondria 242
– – reactions 201, 202, 205, 209, 210, 211, 212, 238 ff
– import, mitochondrial 243
– kinase C 280, 317
– pore-forming 124, 126
– translocation 201, 202
– transport, cytosolic 212 ff
– unfolding 201, 202, 204
– –, energetics 233
proteins, regulatory, thin filament 32 ff
–, secretory 211, 212
purinoceptor agonists, endogenous 297
– –, exogenous 298 ff, 303, 352 ff
purinoceptors, acetylcholine release 350ff
–, prejunctional 303
–, sympathetic transmission 298 ff

rauwolscine 296, 297
receptors, prejunctional, definition 266
–, – functional role 378, 379
–, –, localization 327, 328, 329
–, –, location 378
reticulocyte lysate 222, 232
ribokinase family 68
ribosome receptor 221
ribosomes, nascent chains 230
ribulose bisphosphate carboxylase 243

saralasin 315
sarcomere length 18, 22
– –, cross-bridge interaction 7, 10
sarcoplasmic reticulum, Ca^{2+} pumping 39, 40
senktide 371, 372
signal recognition particle receptor 220
somatostatin content 374
–, GABA interaction 375
–, noradrenaline release 326, 327
–, presence and release 373
– receptors, acetylcholine release 373–375
– terminals, location 374
steroid hormone receptors, hsp90 208
– hormones 224
stiffness, muscle 10, 11
stress response proteins 200
stretch resistance 37
submucous plexus, acetylcholine release 347
substance P 367, 370, 371, 374
–, acetylcholine release 370, 371
–, noradrenaline, release 324
succinate-cytochrome reductase 129, 130
sympathectomy, chemical 279, 283
sympathetic tone, cholinergic influence 281
synaptosomes, acetylcholine release 351

tachykinin receptors, different 370, 371, 372

tachykinins, acetylcholine release 369–372
targeting complex 220
– factor 222
– nuclear proteins 223
TCP complexes 207
temperature, muscle fiber tension 8, 9
tension/pCa relation 8, 12, 32 ff, 48
tension redevelopment 21 ff, 25
thermotolerance, induced 209
thin filament, regulatory subunits 33, 34
– –, actin 2, 3, 16, 32 ff
thioperamide 349
thromboxane, cholinergic transmission 358, 359
– receptor 308, 309
thylakoid membrane, preprotein translocation 234, 235
thyroid hormone, insulin and glucokinase 162
– –, myosin expression 43
titin 37, 38
translocase, hsp70 232, 236
translocation competence 215, 216
–, endoplasmic reticulum, BiP 235, 236
–, energetics 233, 234, 236
–, H^+ gradient 235
–, intermediates 230, 231
– machinery, mitochondrial 227, 228, 229, 231
–, mechanism, hsp70 227–232
–, preprotein conformation 225, 226, 227
–, translation machinery 225
transmitter, overflow 267
– release, intermittency 378
– –, prejunctional modulation 377, 378
trehalose 6-phosphate 70
triglyceride transfer, microsomal 212
tropisetron 347, 348
tropomyosin 2, 3, 15, 33
troponin C 6, 7, 32, 33, 39, 40
–, fast and slow isoforms 36, 37
– isoforms 48
troponin T 33, 34
–, isoforms, effects 34–36
tumors, glycolytic rate 135
twitch tension, post-tetanic potentiation 9

unfoldase 232, 233

vagal nerve stimulation 279, 280, 281, 329
vagosympathetic interaction 279, 280, 281
vasoactive intestinal peptide, NANC transmission 365
VIP, noradrenaline release 326

YDj1p 215, 216, 217
yeast glucokinase 69